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STUDIES ON CARBON DIOXIDE

IV. THE INFLUENCE OF GASEOUS ENVIRONMENT ON GROWTH AND TOXIN PRODUCTION OF C. DIPHTHERIAE[1]

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INTRODUCTION

Since Roux and Yersin discovered the presence of a specific soluble toxin in the culture filtrates of *C. diphtheriae* considerable effort has been made to determine the factors underlying the formation of the toxin. This problem is not only of pronounced theoretical interest, but also has a very important practical bearing on the commercial production of potent toxin and anti-toxin.

Upon reviewing the available literature concerned with the production of diphtheria toxin, one finds little mention of the influence of gaseous environment on growth and toxin formation, aside from the statement that a rich oxygen supply is highly essential. Roux and Yersin (1890 and 1894), Aronson (1894), Spronck (1895), Park and Williams (1896), Madsen (1897) and Martin (1898) at times observed more rapid and abundant toxin formation in cultures aerated with ordinary air than in those which were incubated under the usual conditions. However, passing a current of air over the cultures also resulted in marked irregularity in the toxicity of the filtrates and caused rapid destruction of the toxin once it was formed. Consequently, aeration of diphtheria cultures with ordinary air was considered more deleterious than beneficial.

That carbon dioxide apparently has no inhibiting effect on

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Loeffler serum cultures of *C. diphtheriae* was shown by Fränkel (1889), who observed that growth was as luxuriant under greatly increased carbon dioxide as in the ordinary air. More recently Lorentz (1923) reported a higher percentage of positive findings by incubating field cultures from suspected diphtheritic patients under added carbon dioxide than was obtained by the usual method.

The favorable influence of carbon dioxide on a certain limited number of bacterial species has been known for some time. The meningococcus was found to grow more luxuriantly in an atmosphere containing from 10 to 20 per cent carbon dioxide than under ordinary environmental conditions, by Cohen and Fleming (1918), Gates (1919) and Phelon, Duthie and M'Leod (1927). Similar observations have been made on the gonococcus by Chapin (1918), and on *Bact. abortus* by Huddleson (1921), and Smith (1924). There has been considerable speculation as to how the favorable influence is exerted; however, but little significance was attached to the carbon dioxide per se. Sierkowski and Zajdel (1924) suggested that carbon dioxide may play a rôle as hydrogen ion regulator in all bacterial cultures. The extensive researches of Valley and Rettger (1926 and 1927) have shown that all bacteria thus far studied by them, including *C. diphtheriae*, are unable to grow in the complete absence of carbon dioxide as such.

These observations, together with the fact that the diphtheria organism is exposed to a more or less carbon dioxide-rich gaseous environment in the nose and throat, suggested that carbon dioxide may play an important rôle in the growth and toxin production of *C. diphtheriae*.

The present investigation is concerned chiefly with the influence of different gas mixtures of nitrogen, oxygen and carbon dioxide on the growth and toxin production of *C. diphtheriae*, with a view to acquiring more complete information regarding the principles governing uniform toxin production.

METHODS

Cultures. The well known Park-Williams No. 8 strain of *C. diphtheriae* was obtained from the Hygienic Laboratory and

maintained on Loeffler's serum according to the method recommended by Gibbs and Rettger (1927). Inoculation of the culture flasks, unless otherwise stated, was accomplished by floating a 4 mm. loopful of the surface growth of an "acclimated" meat-infusion-peptone broth culture on the surface of the medium. This method was found to result in more rapid growth and toxin production than was obtained by adding 1 cc. of broth culture to each flask in the usual manner.

Medium employed for toxin production. The commonly used medium containing beef infusion, 2 per cent Difco-Proteose Peptone and 0.5 per cent NaCl was employed. The final reaction was adjusted to pH 7.5. After clarification by heat, the filtered medium was distributed in 500 cc. Erlenmeyer flasks, 90 cc. to each flask. Final sterilization was accomplished by autoclaving at fifteen pounds extra pressure for fifteen minutes.

The following determinations were made on each test culture after definite periods of incubation:

Hydrogen ion concentration, by the colorimetric method of Clark and Lubs (1917).

Amino-nitrogen, by the Sörensen Formol Titration Method as employed by Slanetz and Rettger (1928). The method is essentially that described by Brown (1923), under "Method B."

Ammonia-nitrogen, by the Van Slyke and Cullen modification of the Folin air current method, as used by Slanetz and Rettger (1928). No attempt was made to distinguish between free ammonia and volatile amines, all volatile alkaline substances occurring in a given test culture being calculated as free ammonia.

Bacterial sediment per 100 cc. of culture, by centrifuging an aliquot portion of culture in a calibrated Hopkins centrifuge tube.

Number of viable organisms per cubic centimeter of culture, by the plating method. Meat-infusion-peptone-glucose agar, the reaction of which had been adjusted to pH 7.5 just before using by the addition of sterile normal NaOH solution, was found to give fairly consistent plate counts. The plates were incubated under an atmosphere containing from 3 to 5 per cent carbon dioxide and 20 per cent oxygen for a period of four days. The colonies were then counted with the aid of a hand lens. Plates

incubated under these conditions yielded higher counts and larger colonies, as a rule, than plates incubated in the usual way.

Preservation of culture filtrates. After carrying out the previously mentioned tests on each culture, 0.5 per cent phenol was added as a preservative. The phenol-treated culture was allowed to stand over night in the ice-chest. On the following morning the supernatant fluid was separated from the sediment by decantation and stored in the ice-chest in tightly stoppered containers.

Toxicity of culture filtrates. a. Intracutaneous method. The intracutaneous test, originated by Römer and Sames (1909) and later developed and applied by Glenny and Allen (1921), was used in determining the toxicity of experimental toxins. Results were recorded in Ln/500 doses. One Ln/500 dose, so far as experiments reported herein are concerned, may be defined as the smallest amount of toxin which when injected intracutaneously, together with $\frac{1}{500}$ unit of antitoxin,[2] caused a small area of necrosis distinguishable five days after the injection.

Briefly, the procedure employed is as follows: A portion of the test toxin was diluted with sterile physiological salt solution so that 0.05 cc. of the resulting solution contained the exact amount of toxin to be injected. To 1 cc. of the diluted toxin contained in a sterile test tube was added 1 cc. of a solution which contained $\frac{20}{500}$ units of antitoxin.[2] One-tenth cubic centimeter of the resulting toxin-antitoxin mixture then contained the desired amount of toxin for injection, and also $\frac{1}{500}$ unit of antitoxin. After standing fifteen minutes at room temperature, exactly 0.1 cc. of the toxin-antitoxin mixture was drawn into a tuberculin syringe equipped with a fine gauge needle and injected into the shaven skin of a white guinea pig, as near the surface as possible. As many as six injections were made at one time on a large pig, allowing a space of at least one inch between the injections. Usually 3 injections were made on each side of the median ventral line.

b. Subcutaneous method (L+dose). The procedure described

[2] One unit of antitoxin in this case is defined as the amount of antitoxin which will exactly neutralize one minimal lethal dose of diphtheria toxin.

in Hygienic Laboratory Bulletin No. 21 (April, 1905), was used in the determination of the L+ dose of toxin.

The reliability of the intracutaneous method of standardizing diphtheria toxin

In undertaking an investigation which requires toxicity determinations on large numbers of experimental toxins, it is necessary to select a method which is economical but at the same time fairly accurate.

The flocculation test described by Ramon (1922) is economical and can be carried out rapidly. However, while Ramon's method may measure the antigenic properties of a mixture of toxin and toxoid, it does not necessarily give results which indicate the toxicity of a toxin solution, and for that reason the test was not employed in this investigation.

Of the various methods which depend upon animal inoculation the intracutaneous test appears to be the most economical and is most rapidly carried out. As to its reliability, Glenny and Allen (1921) and Watson and Wallace (1923) report that the results obtained by the intracutaneous method correlate fairly well with those obtained by the subcutaneous test, although no exact relation appeared to exist between the two. The intracutaneous method has been used by Hartley (1922) and by Watson and Wallace (1924) in determining the toxicity of experimental toxins.

Before adopting the intradermal method in the routine testing of culture filtrates, several tests were made to determine its reliability, and to select what may be termed an "end-point." A definite amount of toxin will produce an area of redness 1 cm. in diameter which disappears shortly after forty-eight hours. A slightly larger amount will produce an area of redness 2 cm. in diameter, followed on the third or fourth day by oedema and slight necrosis at the site of the injection; while a still larger amount will cause a distinct area of necrosis 0.5 to 2.0 cm. in diameter in three to five days. This being the case, it is necessary to decide

upon the character of the reaction which will be considered positive.

TABLE 1

Showing the effect of injecting different amounts of toxin intradermally, together with 1/500 unit of antitoxin

AMOUNTS OF TOXIN INJECTED	REACTION PRODUCED				
	24 hours	48 hours	72 hours	4 days	5 days
cc					
0.0003	Redness 1 cm.	0	0	0	0
0 0004	Redness 1 cm.	Redness 1 cm.	0	0	0
0.0005	Redness 1.5 cm.	Redness 1.5 cm.	Slight edema	Peeling 1 cm.	Peeling 1 cm.
0.00075*	Redness 1.5 cm.	Redness 1.5 cm.	Edema 1 cm.	Necrosis 0.3 cm.	Necrosis 0.5 cm.
0.001	Redness 2 cm.	Edema 2 cm.	Necrosis 0.3 cm.	Necrosis 1 cm.	Necrosis 1 cm.
0.0015	Redness 2 cm.	Edema 2 cm.	Necrosis 1 cm.	Necrosis 2 cm.	Necrosis 2 cm.

* Represents the Ln/500 dose. Peeling means sloughing at the site of the injecting.

TABLE 2

Showing the relative susceptibility of guinea pigs to intracutaneous injection of diphtheria toxin

TOXINS	AMOUNT INJECTED	REACTION OBSERVED ON THE FIFTH DAY	
		Guinea pig a	Guinea pig b
	cc.		
IV-20-9	0.0010	1.0 cm. of necrosis	1.0 cm. of necrosis
IV-10-5	0.00075	0.6 cm. of necrosis	0.5 cm. of necrosis
IV-D-9	0.0015	0.8 cm. of necrosis	0.5 cm. of necrosis
S-C-17	0.0004	1.0 cm. of necrosis	0.5 cm. of necrosis
R-C	0.0005	1.0 cm. of necrosis	0.8 cm. of necrosis
R-S-D	0.0010	0.5 cm. of necrosis	0.5 cm. of necrosis
XIV-D-20	0.0005	0.5 cm. of necrosis	0.6 cm. of necrosis

Table 1 illustrates the effect produced by injecting varying amounts of a given toxin, together with $\frac{1}{500}$ unit of antitoxin, intracutaneously.

The smallest amount of toxin which, when injected intra-cutaneously with $\frac{1}{500}$ unit of antitoxin, causes an area of necrosis 0.5 cm. in diameter, distinguishable five days after the injection, was selected as the end-point. Accordingly one Ln/500 dose of toxin was present in 0.00075 cc. of the above toxin.

In order to determine the relative susceptibility of guinea pigs to intracutaneous injections of toxin, similar amounts of toxin-antitoxin mixtures were injected into the skin of different guinea pigs. A few of the results obtained are recorded in table 2.

TABLE 3

Showing the relation of the Ln/500 dose to the L+ dose

TOXINS	Ln/500 DOSE	L+ DOSE	$\dfrac{Ln/500}{L+}$
	cc.	*cc*	
X–5–5	0.0002	0 16	1–800
X–5–10	0 0002	0 15	1–750
X–30–10	0 0002	0 15	1–750
X–50–10	0.0002	0 14	1–700
XIII–15–10	0 00015	0.15	1–1000
A–8	0.0010	0 60	1–600
Hyg.	0 0003	0.20	1–667
IV–10–5	0 0007	0.42	1–600
XIV–5–20	0.0002	0.14	1–700

Average $\dfrac{Ln/500}{L+} = 1/700$.

The results obtained in the two animals checked fairly closely, on the whole. However, a slight variation in the reaction produced by injecting similar doses of a given toxin did occur in a few instances; this was possibly due either to a slight difference in the susceptibility of the guinea pigs injected, or to leakage at the site of the injection, or to a difference in the depth of the injection. It was found necessary, of course, to make all injections as near the surface of the skin as possible, in order to obtain the characteristic local necrosis, instead of the diffuse type which is more apt to follow the deeper injections.

The toxicity of a number of culture filtrates was determined by the intracutaneous and subcutaneous methods for the purpose of

ascertaining the relation of the Ln/500 dose to the L+ dose. The results are recorded in table 3.

These results show that, while no fixed relation exists between the Ln/500 dose and the L+ dose, the latter is approximately 700 times the former. A certain amount of disagreement between the two tests is to be expected: this may be due to several causes, namely, the nature of the toxin injected, and a difference in the susceptibility of the guinea pigs to both the skin test dose and the L+ dose. That guinea pigs vary in their susceptibility to L+ dose toxins has been shown, among others, by Craw and Dean (1907,) and Südmerson and Glenny (1909).

In view of these observations, the authors feel that the intracutaneous test, as described, may be considered a reliable means of approximating the toxicity of experimental toxins. While the skin test is probably not as accurate as the subcutaneous test (determination of the L+ dose), its advantages with respect to saving in animals, materials and time far outweigh its disadvantages.

DESCRIPTION OF AERATION APPARATUS EMPLOYED IN THIS INVESTIGATION

In order to study the effect of definite gas mixtures on growth and toxin production of *C. diphtheriae*, it was necessary to devise an apparatus whereby cultures could be grown in the different gaseous environments. The use of a sealed container would obviously be unsatisfactory for this purpose, as the oxygen tension would be reduced and the carbon dioxide increased during the growth of the culture, and, hence, no definite equilibrium maintained. Consequently, it seemed prudent to aerate the test cultures with known gas mixtures at such a rate that the gas within the culture flasks would remain uniform as to composition. A diagram of the aeration apparatus used in the succeeding experiments is given in figure 1. Arrows indicate the course of the gas mixture.

Three 500-cc. Erlenmeyer flasks equipped with ordinary cotton plugs and containing 90 cc. of sterile broth were inoculated in the usual way. A special cotton plug bearing two bent glass

FIG. 1
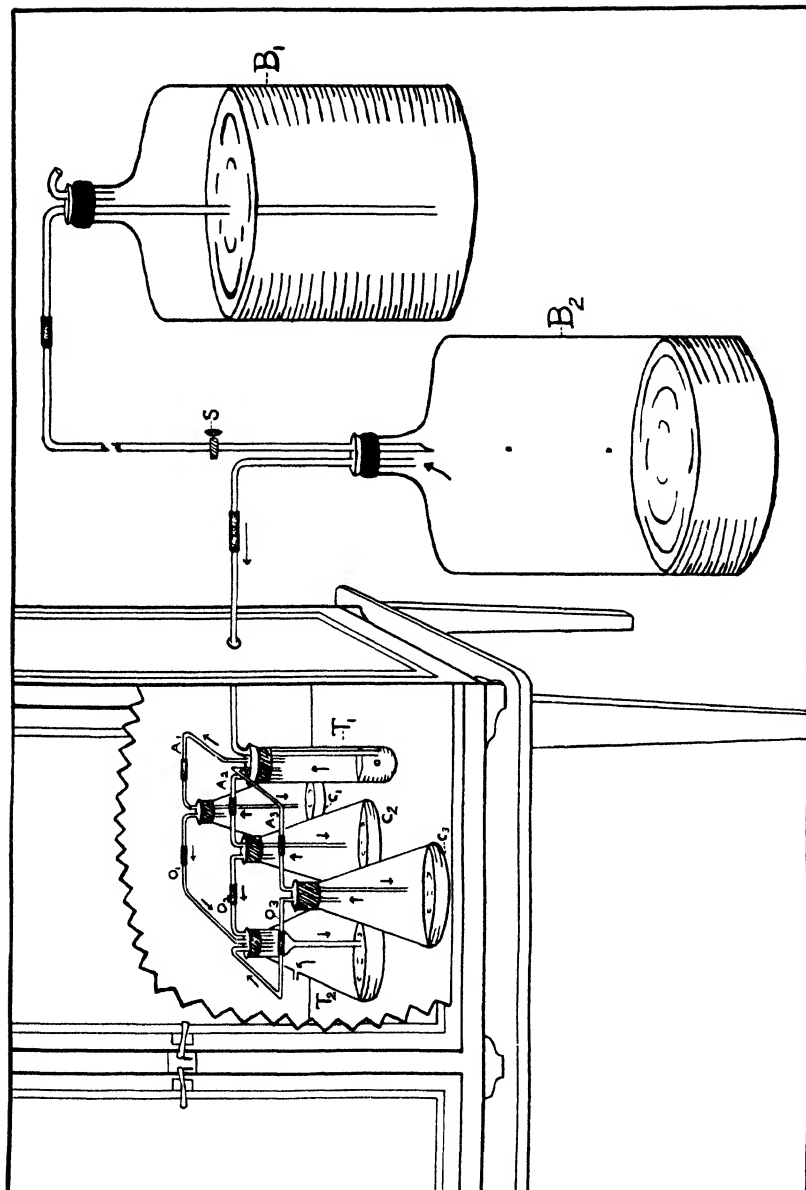
tubes (inlet and outlet tubes) which had been previously wrapped in paper and sterilized in the oven, was then substituted for the ordinary cotton plug in each of the three flasks. The special plugs were pushed down the neck of the culture flasks a distance of 0.5 cm. below the mouth. The flasks were then made airtight, except for the inlet and outlet tubes, by placing a layer of paraffin at least 5 mm. in depth over the special plug. The 3 flasks (C_1, C_2 and C_3) to be aerated were then placed in the system as shown in the accompanying diagram.

A 12-liter bottle (B_1) filled with water or N/100 sulphuric acid, depending on whether or not the test atmosphere contained carbon dioxide, and placed on a shelf at a height of 4 feet over $B_2 \cdot B_1$, was connected with a second 12-liter bottle (B_2) which contained the test gas mixture, by means of suitable glass tubing and a one-way stopcock (S).

The stopcock (S) was then opened and a siphon started between B_1 and B_2 by applying air pressure inside B_1. The rate of flow was regulated by means of stopcock (S) which indirectly governed the rate of gas flow inside the culture flasks. As shown in figure 1, a stream of the test atmosphere flowed from B_2 to a trap (T_1) where it was equally divided between inlet tubes (A_1, A_2 and A_3). In this way separate streams of test gas mixture passed through the culture flasks (C_1, C_2 and C_3). Upon leaving the culture flasks through outlet tubes (O_1, O_2 and O_3) the gas mixture passed through a second trap (T_2) and out into the open air.

The traps (T_1 and T_2) contained 10 per cent sodium hydroxide in instances where the test atmospheres were CO_2-free; at other times they contained 5 per cent sulphuric acid. T_1 served several purposes: (1) it saturated the test atmosphere with moisture, thereby preventing loss of water from the culture; (2) it removed bacteria from the applied gas mixture; (3) it maintained an equal pressure on each pipe line leading to the culture flasks, thereby assuring an even distribution of gas to each culture; and (4) when supplied with 10 per cent sodium hydroxide, it removed traces of carbon dioxide from the test atmospheres. T_2 prevented an inward diffusion of gases from the outside air.

Three aeration systems similar to the one described were set

up in one incubator (large Thelco) and employed throughout the present investigation. The plan and results of the different experiments are presented in the following pages.

Aeration of cultures with carbon dioxide-free air,[3] ordinary air, and air containing 3 per cent CO_2[4]

Sixteen flasks containing 90 cc. of the same lot of meat-infusion-peptone broth were inoculated, each with 1 cc. of a twenty-four-hour culture of *C. diphtheriae*, and placed in the 37°C. incubator. Four of the flasks were aerated with CO_2-free air, four with ordinary air, and four with air containing 3 per cent carbon dioxide; the fourth set of flasks was incubated under ordinary atmospheric conditions. Approximately 100 cc. of test gas mixture were passed over each aerated culture per hour. The results are recorded in table 4.

Toxicity (chart 1). Cultures aerated with air containing 3 per cent carbon dioxide were characterized by greater and more rapid toxin formation than were the cultures incubated under ordinary atmospheric conditions. Maximum toxicity was attained on the fifth day and was maintained at a relatively constant level during the remainder of the twenty-day incubation period. On the other hand, cultures aerated with either CO_2-free air or ordinary air were less toxic than the control cultures and showed a marked irregularity in toxin content. A comparison of chart 4 with chart 1 shows that appreciable amounts of toxin did not appear in any of the cultures until after the period of maximum growth.

Hydrogen ion concentration (chart 2). The hydrogen ion concentration remained within the optimum range for toxin formation (between pH 7.5 and pH 8.0) in the cultures aerated with

[3] Valley and Rettger (1927) have shown that complete removal of carbon dioxide from the culture medium and from the atmosphere in contact with the medium prevents the growth of *C. diphtheriae*. In this experiment no attempt was made to remove carbon dioxide from the culture medium. As growth was obtained in the cultures aerated with CO_2-free air, it must be assumed that sufficient CO_2 was dissolved in the medium to initiate growth.

[4] The results reported in this experiment have been presented in a preliminary report by Plastridge and Rettger (1927).

air containing 3 per cent carbon dioxide.   On the other hand, the pH values of the cultures aerated with ordinary air and CO_2-free air increased from pH 7.5 to pH 9.0 by the fifth day of incubation and remained at pH 9.0 throughout the remainder of the incubation period.   The increase in pH of the control cultures followed

TABLE 4

Showing the results of aeration with CO_2-free air, ordinary air, and air containing 3 per cent CO_2, as compared with those obtained under ordinary conditions

CULTURE	DAYS INCUBATED 37°C.	Ln/500 DOSE	pH	NH₃-N in 100 cc.	SEDIMENT IN 100 cc.	GROWTH	VIABLE CELLS PER CUBIC CENTIMETER
		cc.		*cc.*	*cc.*		
Controls incubated under ordinary conditions	3	0.01	8.6	27.8	1.1	M. C.	250,000,000
	5	0.003	8.6	26.2	1.2	M. C.	325,000
	8	0.0018	8.9	23.3	1.1	H. C.	2,200
	20	0.005	8.9	13.4	0.8	M. C.	60,000
Cultures aerated with CO_2-free air	3	0.01	8.4	28.7	1.2	H. C.	360,000,000
	5	0.0025	8.8	29.0	1.2	H. C.	257,000
	8	0.0025	9.0	28.0	1.0	Br. C.	2,200
	20	0.005	9.0	27.0	1.0	Br. C.	1,000
Cultures aerated with ordinary air	3	0.01	8.4	27.7	1.2	H. C.	63,000,000
	5	0.01	9.0	29.1	1.2	H. C.	26,000
	8	0.004	9.0	28.0	1 1	Br. C.	3,000
	20	0.008	9.0	26.0	1.1	Br. C.	8,000
Cultures aerated with air containing 3 per cent CO_2	3	0.01	7.8	29.0	1.2	H. C.	315,000,000
	5	0.0008	7.8	30.7	1.2	H. C.	227,000,000
	8	0.0008	8.0	31.1	1.2	H. C.	95,000,000
	20	0.001	8.0	31.7	1.2	Br. C.	13,000,000
Medium			7.5	7.0			

M. C. = moderate crust; H. C. = heavy crust; Br. C. = broken and partly sunk crust.

somewhat the same general course, except that alkalinization was more gradual, the maximum being reached on the eighth day.

Ammonia production (chart 3).   The ammonia content of all cultures showed a sharp increase during the first three days of
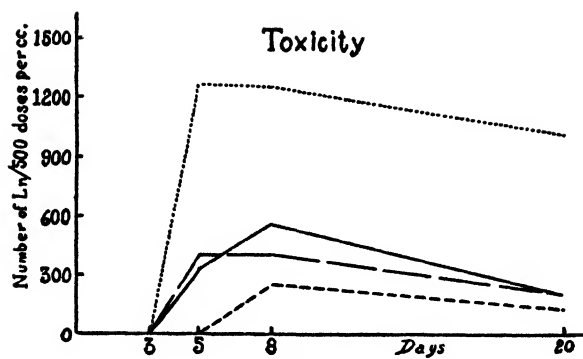
CHART 1
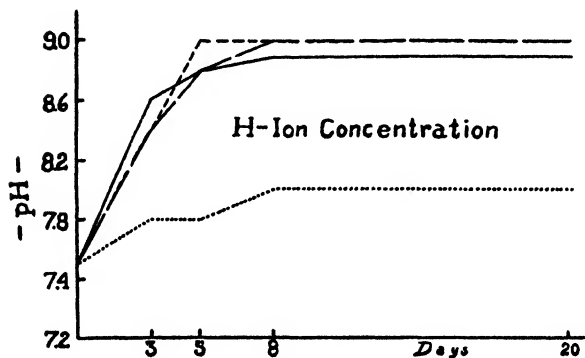
CHART 2
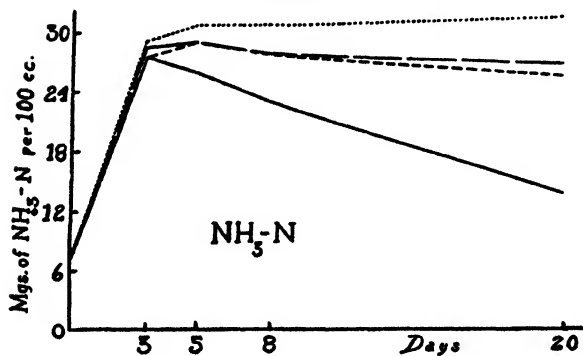
CHART 3

——————— control.
— — — aerated with ordinary air.

incubation. In the control cultures and in those aerated with CO_2-free air or ordinary air this increase was followed by a gradual decline, while a continuous increase occurred in the flasks aerated with air containing 3 per cent carbon dioxide. Apparently the ammonia content of cultures grown under an increased carbon dioxide tension is an indication of the pronounced peptolytic activity of the diphtheria organism.

Plate count (chart 4). The number of viable organisms per cc. in all cultures reached a maximum during the first three days of incubation. In the cultures aerated with an atmosphere containing 3 per cent carbon dioxide the plate count remained at a
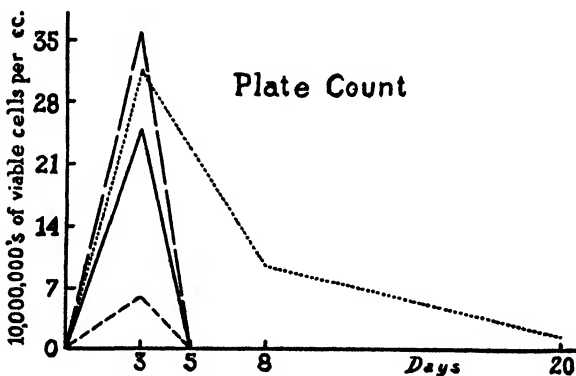
CHART 4

---------- aerated with CO_2-free air.
.... aerated with air + 3 per cent CO_2.

comparatively high figure during the entire twenty-day incubation period. On the other hand, a sharp drop occurred immediately after the maximum count was reached in the control cultures, and in the cultures aerated with either CO_2-free air or ordinary air; this is presumably due to the marked increase in alkalinity of the culture medium during the period of maximum growth.

Bacterial sediment. The amount of bacterial sediment produced in the cultures aerated with air containing 3 per cent carbon dioxide surpassed that of the controls. A maximum was reached during the first three days of growth, which remained unchanged

during the remainder of the twenty-day incubation period. Aeration of the cultures with CO_2-free air or ordinary air, under the conditions of the experiment, also resulted in increased rate of growth during the first three days of incubation.

Summary. The results presented here show that carbon dioxide plays an important rôle in the accumulation of toxin in cultures of *C. diphtheriae*. Under an atmosphere of air containing 3 per cent carbon dioxide toxin formation was greatly accelerated and the total amount of toxin accumulating in a given culture was much greater than in a culture grown under the usual conditions, or aerated with either CO_2-free air or ordinary air. Furthermore, an increased carbon dioxide tension over the culture medium apparently prevented, to a large extent, destruction of the toxin once it was formed.

Aeration of broth cultures of *C. diphtheriae* with an atmosphere containing 3 per cent carbon dioxide also favored abundant growth and prevented the usual rapid destruction of the bacterial cells after the period of maximum growth.

Aeration of cultures with atmospheres containing different concentrations of carbon dioxide

In order to determine the carbon dioxide tension most conducive to maximum growth and toxin accumulation, broth cultures of *C. diphtheriae* were aerated with atmospheres containing different percentages of carbon dioxide and approximately 18.5 per cent oxygen.

Twelve flasks of peptone-meat-infusion bouillon were prepared, using Difco-Proteose Peptone, and inoculated with 1 cc. of broth culture of *C. diphtheriae*. These culture flasks were divided into 4 groups, 3 flasks in each group. Group I was incubated under ordinary atmospheric conditions and served as the control, while Groups II, III and IV were aerated with atmospheres containing 5, 10 and 20 per cent carbon dioxide, respectively. One flask was removed from each group after five, nine and twenty days incubation, and the usual determinations made on each. The results are recorded in table 5.

Toxicity (chart 5). The toxicity of cultures grown under a gas mixture containing 10 per cent carbon dioxide and 18.5 per cent oxygen was somewhat greater than that of those grown under atmospheres containing either 5 or 20 per cent carbon dioxide. However, all cultures grown under an increased carbon dioxide tension were more toxic than the controls. No significant deterioration of toxin occurred in the cultures which were incu-

TABLE 5

Showing the influence of different amounts of carbon dioxide

CULTURE	NUM-BER OF DAYS INCU-BATION	Ln/500 DOSE	pH	AMINO-N PER 100 cc.	NH₃-N PER 100 cc.	SEDI-MENT IN 100 cc.	CHARAC-TER OF GROWTH	VIABLE CELLS PER CUBIC CENTIMETER
		cc.		mgm.	mgm	cc.		
Controls incubated under ordinary conditions	5	0.0017	9.1	73.0	16.5	0.95	M. C.	536,000
	9	0.0015	9.1	79.4	14 2	1.00	M. C.	56,000
	20	0 0025	9.0	77.1	8.0	0 95	M. C.	0
Aerated with air containing 5 per cent CO₂	5	0.001	8.0	79.5	24 8	1.2	H. C.	51,000,000
	9	0.001	8.1	80 4	28.4	1.2	H. C.	40,000,000
	20	0.001	8.2	74.1	34 1	1.2	H. C.	4,800,000
Aerated with air containing 10 per cent CO₂	5	0.0007	7.9	76.9	25.3	1.0	H. C.	65,000,000
	9	0.0008	7.8	78.0	30.1	1.1	H. C.	73,000,000
	20	0.0008	8.0	72 7	36.9	1.1	H. C.	7,500,000
Aerated with air containing 20 per cent CO₂	5	0.0015	7.6	74 5	27.1	0.9	M. C.	110,000,000
	9	0.001	7.4	79.2	30.5	0.95	H. C.	91,000,000
	20	0.001	7.6	71.9	36.2	1.0	H. C.	21,000,000
Medium			7.5	64.0	10.0			

M. C. = moderate crust; H. C. = heavy crust.

bated under atmospheres containing 5, 10 or 20 per cent carbon dioxide.

Hydrogen ion concentration (chart 6). The final pH values of the control cultures and the culture incubated under gas mixtures containing 5, 10 and 20 per cent CO_2 were 9.0, 8.2, 8.0 and 7.6, respectively. If the favorable effects of an increased carbon dioxide tension on toxin production and growth is due principally
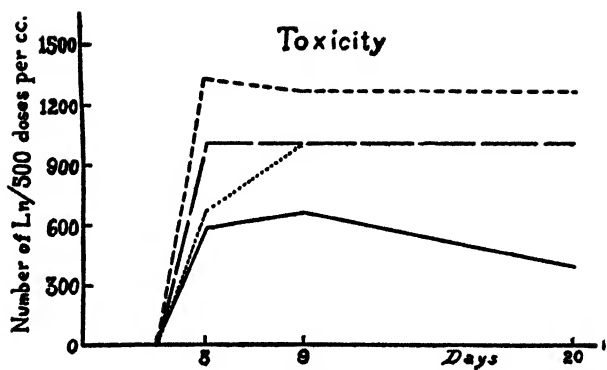
CHART 5
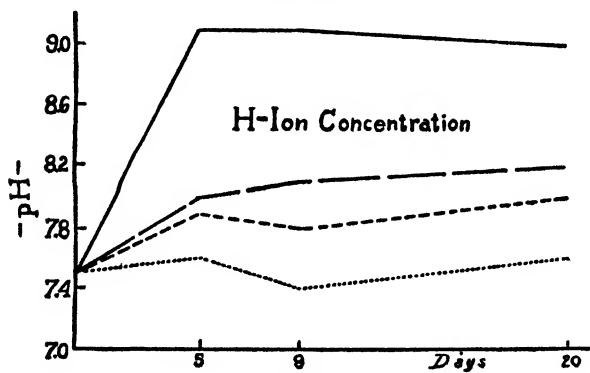
CHART 6
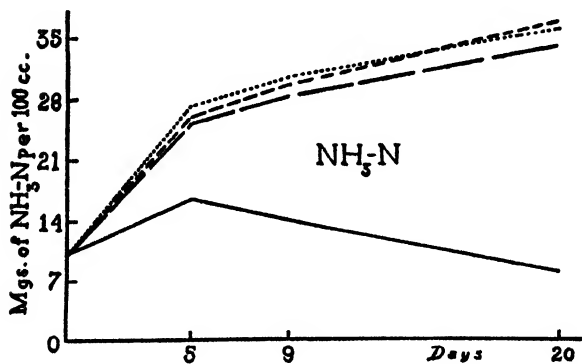
CHART 7

——————— control.
— — — aerated with 5 per cent CO_2.

to a regulation of the reaction of the culture medium, these results would show that a maintained hydrogen ion concentration of from pH 7.8 to 8.0 is the optimum for growth and toxin accumulation.

Ammonia nitrogen (chart 7).   The accumulation of ammonia in the control cultures (unaerated flasks) was more gradual and never as great as in the other cultures; furthermore, a slow but continuous decrease in ammonia content occurred between the end of the period of maximum growth and the end of the 20 day incubation period.   Apparently, under the usual conditions of
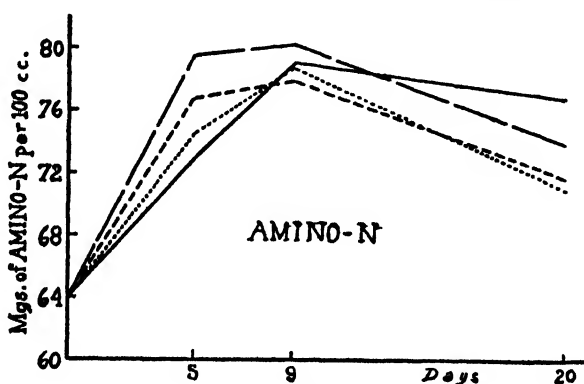
CHART 8

---------- aerated with 10 per cent CO_2.
.... aerated with 20 per cent CO_2.

artificial cultivation, ammonia passed off into the outside atmosphere, but was retained in the cultures aerated with an atmosphere containing from 3 to 20 per cent carbon dioxide.   The presence of appreciable numbers of viable cells during the entire twenty-day incubation period in cultures grown under an increased carbon dioxide tension probably explains the continuous increase in ammonia in such cultures.

Amino nitrogen (chart 8).   Increase in amino nitrogen was more rapid in cultures aerated with atmospheres containing from 5 to 20 per cent carbon dioxide than in the control cultures.   Subsequent decrease in amino nitrogen content was more marked in the former than in the latter, a condition which shows that an

increased carbon dioxide tension within certain limits increases either the amount or the activity of the peptolytic and deaminizing enzymes of *C. diphtheriae*.

Plate count (chart 9).   The number of viable organisms remaining after the period of maximum growth was considerably greater in the cultures aerated with atmospheres containing either 5, 10 or 20 per cent carbon dioxide, particularly the last, than in the control cultures.   The plate count for the control cultures approached zero on the fifth day of growth; as no counts were
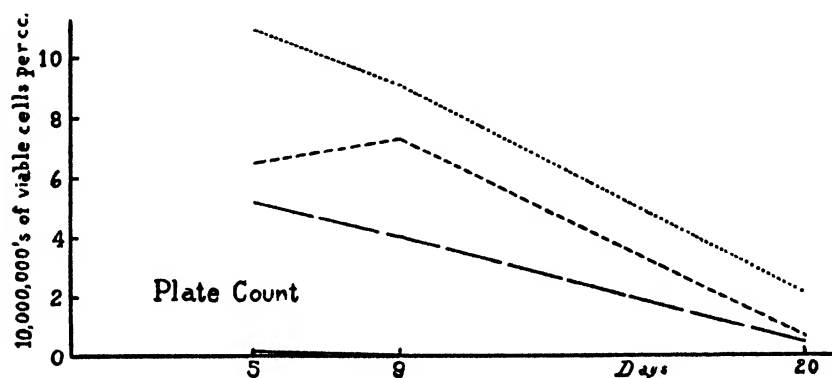
CHART 9
——————— control.
—— —— aerated with 5 per cent CO_2.
---------- aerated with 10 per cent CO_2.
. . . . aerated with 20 per cent CO_2.

made before the fifth day, the curve for the control cultures is barely visible in chart 9.

Bacterial sediment.   More bacterial sediment was produced in the cultures aerated with an atmosphere containing from 5 to 10 per cent carbon dioxide than in any of the other cultures.

Remarks.   From these results the conclusion may be drawn that from 5 to 10 per cent carbon dioxide within the culture flasks is the optimum concentration for growth and toxin accumulation.

Growth and toxin production in bouillon prepared with Difco-Bacto Peptone

The character of the "peptone" employed in the production of diphtheria toxin has been known for some time to have a direct bearing on the quantity of toxin obtained in culture filtrates. Consequently, many attempts have been made to determine why certain "peptones" favor abundant toxin accumulation while other "peptones" yield little or no toxin, even though they support abundant growth of *C. diphtheriae*. The results of past investigation have led to the development of the theory that simple nitrogenous compounds such as amino-acids are necessary and sufficient for growth, while more complex bodies, probably proteoses, are essential for toxin accumulation.

As to how these proteose fractions come into play in toxin formation has been a matter of considerable speculation. Dernby and Walbum (1923) suggest that the diphtheria organism elaborates an enzyme which converts certain proteoses present in the culture medium into the specific toxic substance found in cultures of *C. diphtheriae*. Kligler (1917) and Bunker (1919) intimate that "peptones" of high proteose content are necessary for toxin production because of their buffering effect on the reaction of the culture medium.

The experiments already described show that it is possible to regulate the hydrogen ion concentration of the culture medium (within limits) by increasing the carbon dioxide tension over the culture. Difco-Proteose Peptone is especially adapted for toxin production, and is known to possess a high proteose content. On the other hand, Difco-Bacto Peptone, while it will support abundant growth of most bacteria, is known to be quantitatively lacking in higher protein derivatives.[5] Therefore, in order to determine whether or not the proteose fractions of a "peptone" are necessary for toxin formation purely because of their buffering action, the following experiment was carried out, employing Difco-Bacto Peptone in place of Difco-Proteose Peptone.

[5] A chemical analysis of these two peptones has been reported by McAlpine and Brigham (1928).

Twelve flasks of bouillon were prepared with Difco-Bacto Peptone. These were inoculated, each with 1 cc. of an acclimated culture of *C. diphtheriae*, and divided into 4 groups, 3 flasks in each group. Group I served as the control, while Groups

TABLE 6

Giving the results obtained with Difco Bacto Peptone under different concentrations of carbon dioxide

CULTURE	NUMBER OF DAYS INCUBATED	Ln/500 DOSE	pH	AMINO-N IN 100 cc	NH₃-N IN 100 cc	NUMBER OF CUBIC CENTIMETERS SEDIMENT IN 100 CC.	CHARACTER OF GROWTH	VIABLE CELLS PER CUBIC CENTIMETER
		cc		*mgm*	*mgm*			
Set I. Incubated	5	>0 05	9 0	77.2	20.4	0 8	L. C.	30,000,000
under ordinary	9	0 05	9 0	78 1	15 4	0 8	L. C.	12,000
conditions	20	>0 05	8 8	80 1	14 1	0 7	Br. C.	10,000
Set II. Aerated	5	>0 05	7 8	71 5	27.1	0 8	M. C.	40.000,000
with air containing	9	0 05	8 0	75 2	30 0	1 0	M. C.	30,000,000
5 per cent CO₂	20	0 05	8 0	71 0	31 2	1 0	M. C.	3,100,000
Set III. Aerated	5	0.03	7 6	72 6	30 0	0 9	M. C.	65,000,000
with air containing	9	0 03	7 6	74 4	30 8	1.0	M. C.	24,000,000
10 per cent CO₂	20	0.03	7.7	70 5	31 4	1 0	M. C.	5,500,000
Set IV. Aerated	5	>0 05	7.3	70 8	28 6	0 8	L. C.	75,000,000
with air containing	9	>0 05	7 2	67.7	31.1	0 95	M. C.	40,000,000
20 per cent CO₂	20	>0 05	7.2	76 1	32 1	1 0	M. C.	10,000,000
Medium			7 5	68 8	8 0			

Note: (>) means that the Ln/500 dose is greater than the amount of toxin given in the table. The amounts of toxin preceded by this sign failed completely to give a reaction.

L. C. = light crust; M. C. = moderate crust; Br. C. = broken crust partly sunk.

II, III and IV were aerated with atmospheres containing 5, 10 and 20 per cent carbon dioxide, respectively. One flask was removed from each group after 5, 9 and 20 days of incubation.

The results are recorded in table 6.

Toxicity. While all cultures grown on Difco-Bacto Peptone

were but feebly toxic, toxin formation was most rapid and abundant in cultures aerated with an atmosphere containing 10 per cent carbon dioxide. No decrease in toxicity occurred in these cultures on prolonged incubation. In the control cultures, a trace of toxin appeared on the ninth day and disappeared after 20 days incubation. No toxin was detectable at any time in the cultures aerated with an atmosphere containing 20 per cent carbon dioxide, probably because the hydrogen ion concentration of these cultures was unfavorable for toxin accumulation (pH 7.2).

Growth. Under similar conditions of incubation, less growth was obtained with Difco-Bacto Peptone than with Difco-Proteose Peptone, as shown by bacterial sediment determinations, even though surface growth was abundant in nutrient broth prepared with either "peptone." There was not enough difference in growth, however, in the two media to explain the marked difference in toxin accumulation. Maximum growth occurred in cultures grown under from 5 to 10 per cent carbon dioxide.

Ammonia nitrogen. Ammonia production was more rapid in broth made with Difco-Bacto Peptone than in broth made with Difco-Proteose Peptone. However, the total amount produced was greater in broth prepared with the latter. Maximum ammonia accumulation occurred in cultures aerated with an atmosphere containing either 5 or 10 per cent carbon dioxide.

Hydrogen ion concentration. The hydrogen ion concentration of cultures subjected to 5, 10 and 20 per cent carbon dioxide was maintained at pH 8.0, pH 7.6 and pH 7.2, respectively.

Amino nitrogen. An increase in amino nitrogen occurred in all cultures during the first nine days of growth, which was followed by a gradual decrease throughout the remainder of the incubation period in cultures aerated with an atmosphere containing from 5 to 10 per cent carbon dioxide. In some of the control cultures the usual decrease in amino nitrogen content failed to occur, as shown in table 6. This condition was the exception rather than the rule.

A comparison of the change in amino nitrogen content of cultures grown in broth prepared with Difco-Bacto Peptone and in broth prepared with Difco-Proteose Peptone is given in chart

10. A relatively slight increase in amino nitrogen occurred in cultures in which Difco-Bacto Peptone was employed, as compared with a marked increase in cultures grown in Difco-Proteose Peptone broth. Apparently protein derivatives which can be broken down into amino acids by the peptolytic enzymes of *C. diphtheriae* are present in much smaller amounts in Difco-Bacto Peptone than in Difco-Proteose Peptone.

Remarks. From these results it appears that the proteose fractions of certain "peptones" suitable for diphtheria toxin production are primarily necessary for reasons other than their buffering effect on the reaction of the culture medium. While a
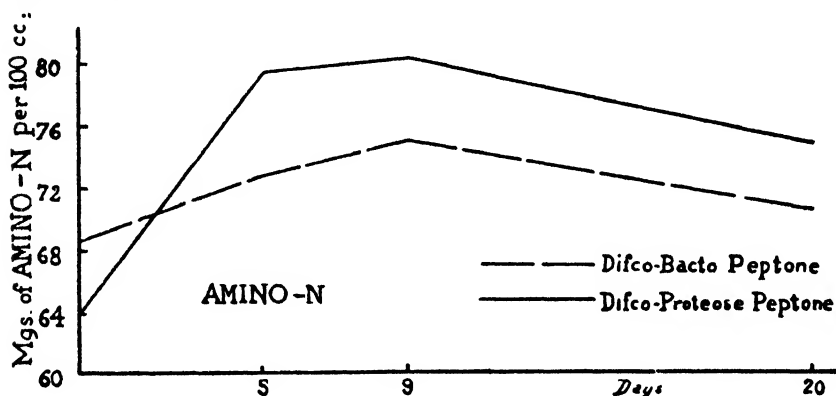
CHART 10

rich supply of proteose is not essential for development, growth is more luxuriant in a medium containing relatively large amounts of higher protein derivatives.

The influence of different oxygen tensions on growth and toxin production

An abundant oxygen supply has been regarded as essential for toxin production for some time, although few attempts have been made to determine the effect of different oxygen tensions on the growth and toxin production of *C. diphtheriae*.

Wherry (1917) found that the oxygen supply is an important factor in the evolution of the larger types of *C. diphtheriae*, low

oxygen tensions favoring the formation of barred forms, and distinctly aerobic conditions favoring the formation of small solid-staining types. Diphtheria organisms re-isolated from guinea pigs which had been injected with virulent strains of *C. diphtheriae* grew better at lowered oxygen tension than under ordinary atmospheric conditions.

Lorentz (1923) observed that colonies of *C. diphtheriae* grown on Loeffler's serum under pure oxygen were smaller and fewer in number than those grown under ordinary aerobic conditions. Under an atmosphere of pure oxygen the individual cells were
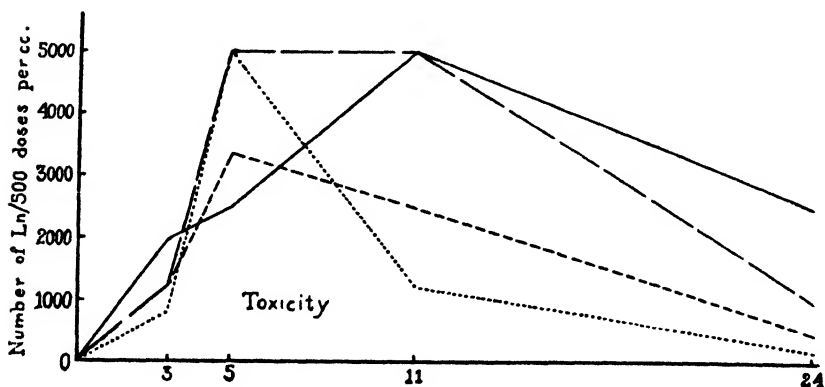
CHART 11. IN THE ABSENCE OF ADDED CO_2

shorter and thicker than in the control cultures, and showed a marked reduction in polar granules. He further observed that cultures grown under pure oxygen gas were more toxic than those grown under pure carbon dioxide gas.[6]

As it is possible to maintain fairly constant oxygen and carbon dioxide tensions in culture flasks by means of the aeration system described in this paper, a study was made of the influence of different oxygen tensions in the presence and in the absence of added carbon dioxide upon growth and toxin production of *C. diphtheriae*.

[6] Diphtheria toxin is never found in media having an acid reaction. As our results have shown, more than 20 per cent carbon dioxide in the atmosphere over the culture inhibits toxin formation through the resulting increase in hydrogen ion concentration of the medium.

A. Cultures grown under increased oxygen tensions. 1. In the absence of added CO_2. Sixteen flasks of broth were prepared in

TABLE 7

Showing influence of increased oxygen in the absence of added carbon dioxide

CULTURE	NUMBER OF DAYS INCUBATION	Ln/500 DOSE	pH	NH_3-N IN 100 cc OF CULTURE	SEDIMENT IN 100 cc OF CULTURE	CHARACTER OF GROWTH	NUMBER OF VIABLE CELLS PER CUBIC CENTIMETER OF CULTURE
		cc.		*mgm*	*cc.*		
Group I. Controls. Incubated under ordinary conditions	3	0 0005	7 9	20 3	0 8	M. C.	135,000,000
	5	0 0004	9 1	23 0	1.2	M. C.	1,500,000
	11	0 0002	9 1	12 6	1 1	M. C.	No colony 1/10 plate
	24	0 0004	9 0	8 8	1 1	M. C.	No colony 1/10 plate
Group II. Aerated with gas-mixture containing 21 per cent oxygen	3	0 0008	8 0	24 5	0 7	M. C.	117,000,000
	5	0 00018	8 4	24 0	0 9	M. C.	6,000,000
	11	0 0002	8 4	26 8	1 0	M. C.	No colony 1/10 plate
	24	0 001	9 1	15.1	1 0	Br. C.	No colony 1/10 plate
Group III. Aerated with a gas-mixture containing 30 per cent oxygen	3	0 0008	8 1	21 0	0.5	L. C.	83,000,000
	5	0 0003	9 0	25 7	0 8	M. C.	300,000
	11	0 0004	9 1	22 7	1 0	M. C.	No colony 1/10 plate
	24	0 002	9 1	13 9	1 0	M. C	No colony 1/10 plate
Group IV. Aerated with a gas-mixture containing 50 per cent oxygen	3	0 0012	8 0	20 5	0 5	L. C.	60,000,000
	5	0 0002	8 8	27.0	1 0	M. C.	2,000,000
	11	0 0008	9.1	19 2	1 0	M. C.	No colony 1/10 plate
	24	0 005	9 1	12.4	1 0	Br. C.	No colony 1/10 plate
Medium			7 5	9.5			

M. C. = Moderate crust; L. C. = light crust; Br. C. = broken crust partly sunk.

the usual way with Difco-Proteose Peptone, and inoculated in the adopted fashion. Four of the flasks were incubated under

ordinary atmospheric conditions as controls; 4 were aerated with CO_2-free atmosphere, containing 21 per cent oxygen; 4 were similarly exposed to 30 per cent oxygen; and 4 to a CO_2-free atmosphere containing 50 per cent oxygen. One flask was removed from each group after three, five, eleven and twenty-four days incubation. The usual determinations were made on each culture.

The results are recorded in table 7.

Toxicity (chart 11). Toxin formation was most rapid during the first three days of incubation in the cultures grown under ordinary atmospheric conditions. This was undoubtedly due to the fact that CO_2-free atmospheres were passed over the aerated cultures. However, after initial growth was once started, this condition was reversed, the five-day aerated cultures being more toxic than the five-day controls. Maximum potency was attained in the aerated cultures on the 5th day, and in the controls on the 11th day.

A rapid decrease in toxin content of the aerated cultures occurred immediately after maximum potency was reached. The rate of decrease was greatest in cultures treated with 50 per cent oxygen, slightly less rapid in those aerated with an atmosphere containing 30 per cent oxygen, still less rapid in cultures under 21 per cent oxygen, and least rapid in the control cultures. The number of viable organisms per cubic centimeter of test culture decreased rapidly after the period of maximum growth, both in the control and in the aerated cultures.

2. In the presence of 5 per cent CO_2. The preceding experiment was repeated, except that test atmospheres of 5 per cent carbon dioxide were employed with the 21, 30 and 50 per cent oxygen, respectively.

The results are recorded in table 8.

Toxicity (chart 12). Toxin accumulation was more rapid and abundant in the cultures aerated with an atmosphere containing 5 per cent carbon dioxide and 21 per cent oxygen than in either the controls or the cultures exposed to 5 per cent carbon dioxide and either 30 or 50 per cent oxygen.

No decrease in toxicity occurred in cultures aerated with a

gas-mixture containing 5 per cent carbon dioxide and 21 per cent oxygen on prolonged cultivation. A slight loss in potency occurred at the end of twenty-four days incubation in cultures

TABLE 8

Showing the influence of increased oxygen supply in the presence of 5 per cent carbon dioxide

CULTURE	NUMBER OF DAYS INCUBATION	Ln/500 DOSE	pH	NH$_3$-N IN 100 CC OF CULTURE	SEDIMENT IN 100 CC. CULTURE	CHARACTER OF GROWTH	NUMBER OF VIABLE CELLS PER CUBIC CENTIMETER OF CULTURE
		cc.		mgm	cc.		
Group I. Controls. Incubated under ordinary atmospheric conditions	3	0.0005	7.9	20 3	0.6	M. C.	120,000,000
	5	0.0004	9.1	21.7	0 9	M. C.	1,000,000
	10	0 0003	9.1	14.0	1 0	M. C.	No colony 1/10 plate
	24	0.0015	9.0	6.0	0.9	M. C.	No colony 1/10 plate
Group II. Aerated with a gas-mixture containing 20 per cent oxygen and 5 per cent CO$_2$	3	0 0002	8 0	23.0	1.0	H. C.	150,000,000
	5	0.0002	8 0	30 1	1 0	H. C.	33,000,000
	10	0 0002	8.1	33 4	1.0	H. C.	64,000,000
	24	0.0002	8.1	36 0	1 0	H. C.	1,600,000
Group III. Aerated with a gas-mixture containing 30 per cent oxygen and 5 per cent CO$_2$	3	0.0005	8.0	21 7	0 95	H. C.	118,000,000
	5	0 0002	8 0	31.7	1.0	H. C.	18,000,000
	10	0.0002	8 0	32 6	1 0	H. C.	2,000,000
	24	0.00025	8.1	33.0	1.1	H. C.	500,000
Group IV. Aerated with a gas-mixture containing 50 per cent oxygen and 5 per cent CO$_2$	3	0 0008	7.6	20 3	0 6	M. C.	135,000,000
	5	0.0002	7 8	30 5		M. C.	62,000,000
	10	0 0002	8.1	31 5	0 8	M. C.	1,200,000
	24	0.0004	8.1	32.7	0.9	M. C.	40,000
Medium			7 5	10.1			

M. C. = moderate crust; H. C. = heavy crust.

grown under an increased oxygen tension; however, this decrease in toxicity was not nearly as great in the presence, as in the absence, of added carbon dioxide.

Growth. The number of viable organisms remaining after the

period of maximum growth was highest in cultures grown under an atmosphere containing 5 per cent carbon dioxide and 21 per cent oxygen, although the number remaining in all cultures aerated with test atmospheres containing added carbon dioxide was appreciably greater than the number remaining in the control cultures.

Sediment determinations and the character of the surface growth showed that growth was most rapid and abundant in cultures aerated with an atmosphere containing 5 per cent carbon
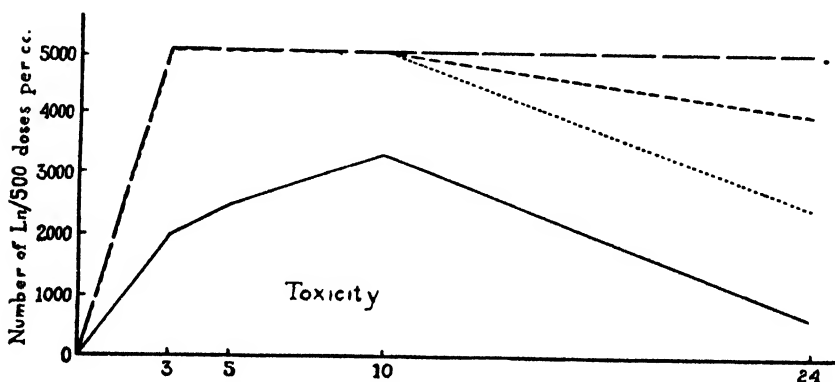
CHART 12. IN THE PRESENCE OF 5 PER CENT CO₂

——————— control.
—— —— oxygen tension—21 per cent.
---------- oxygen tension—30 per cent.
.. .. oxygen tension—50 per cent.

dioxide and 21 per cent oxygen, while growth was retarded and least abundant in cultures grown under a gas-mixture containing 5 per cent carbon dioxide and 50 per cent oxygen.

Remarks. An increased oxygen tension above 21 per cent, in the absence of added carbon dioxide, retards the growth of *C. diptheriae* and causes increased irregularity in toxin accumulation. These unfavorable conditions are offset to a large degree by the presence of added carbon dioxide.

From the results presented, the conclusion may be drawn that 21 per cent oxygen in the atmosphere over *C. diphtheriae* cultures is more favorable for growth and toxin accumulation than higher oxygen tensions.

B. Cultures grown under reduced oxygen tensions. Obviously, the oxygen concentration prevailing under natural conditions of growth for *C. diphtheriae* is less, and the carbon dioxide tension greater, than that prevailing under ordinary aerobic conditions of artificial cultivation. At the same time, the results of the foregoing experiment show that an increased oxygen tension above 21 per cent does not favor growth and toxin accumulation. In view of these facts, the following experiment was conducted for the purpose of determining the optimum and minimum oxygen tensions for growth and toxin production, and to determine whether or not added carbon dioxide influences growth and toxin accumulation in cultures grown under reduced oxygen supply.

Lowered oxygen tensions were obtained by collecting the required amounts of air, carbon dioxide and nitrogen gas[7] over water in the gas supply bottles of the aeration systems. Samples of the resulting gas-mixtures were analyzed by means of an Orsat-Lunge Gas Apparatus.

1. In the absence of added CO_2. Sixteen flasks of meat-infusion-peptone broth were inoculated with *C. diphtheriae*, divided equally into 4 groups, and placed in the incubator. Three of the groups were aerated with atmospheres containing 15, 10 and 5 per cent oxygen, respectively, while the remaining group was grown under ordinary atmospheric conditions and served as the control. One flask was removed from each group after three, five, ten and twenty days incubation.

The results are tabulated in table 9.

Toxicity (chart 13). During the first three days of growth the rate of toxin formation in the cultures aerated with atmospheres containing either 10 or 15 per cent oxygen paralleled that of the control cultures, while toxin production was somewhat retarded in cultures aerated with a gas mixture containing 5 per cent oxygen.

Maximum potency was reached in all cultures on the fifth day of incubation. Toxin accumulation was greatest in cultures

[7] The nitrogen used for this purpose was prepared in the laboratory as needed, by heating the proper mixture of ammonium sulphate and sodium nitrite in aqueous solution.

aerated with an atmosphere containing 15 per cent oxygen, slightly less in cultures supplied with 10 per cent oxygen, still

TABLE 9

Showing the influence of reduced oxygen tension in the absence of added carbon dioxide

CULTURE	DAYS INCU-BATION	Ln/500 DOSE	pH	NH₃-H IN 100 CC.	SEDI-MENT IN 100 CC. CUL-TURE	CHARAC-TER OF GROWTH	VIABLE CELLS PER CUBIC CENTIMETER CULTURE
		cc.		*mgm.*	*cc.*		
Group I. Controls, grown under ordinary atmospheric conditions	3	0.0003	8.6	20 1	0.9	L. C.	250,000,000
	5	0 00025	8 8	21.7	0.9	M. C.	300,000
	10	0.00025	9.0	13 0	0.8	M. C.	No colony 1/10 plate
	20	0 0005	9 0	8.7	0.8	M. C.	No colony 1/10 plate
Group II. Aerated with CO₂-free atmosphere containing 15 per cent oxygen	3	0.0003	7.8	13.2	0 9	L. C.	250,000,000
	5	0 00015	8.6	22 2	1 0	H. C.	47,000,000
	10	0.0002	8 9	23 1	1 0	H. C.	No colony 1/10 plate
	20	0.0003	9.1	14.7	0 9	H. C.	No colony 1/10 plate
Group III. Aerated with CO₂-free atmosphere containing 10 per cent oxygen	3	0 00025	7.6	20.3	1 0	L. C.	300,000,000
	5	0.00018	8.2	22 0	1 0	H. C.	53,000,000
	10	0.00025	9.0	20.3	0.9	M. C.	No colony 1/10 plate
	20	0.0003	9 1	16.4	0.85	M. C.	No colony 1/10 plate
Group IV. Aerated with CO₂-free atmosphere containing 5 per cent oxygen	3	0.001	7.1	18.6	0 6	L. F.	210,000,000
	5	0 0002	8 2	21.4	1.0	H. C.	113,000,000
	10	0.0002	8 8	21.4	0 8	M. C.	No colony 1/10 plate
	20	0.0002	8.9	11.6	0.9	M. C.	No colony 1/10 plate
Medium			7.5	10.6			

L. C. = light crust; M. C. = moderate crust; H. C. = heavy crust; L. F. = light film.

less in cultures grown under a gas mixture containing 5 per cent oxygen, and least in the control cultures.

Decrease in toxicity after the period of maximum potency was inversely proportional to the oxygen tensions in the culture flasks. No noticeable decrease in toxicity occurred in the twenty-day cultures grown under an atmosphere containing 5 per cent oxygen; there was a marked decrease in toxicity in cultures grown under an atmosphere containing 10 per cent oxygen; the decrease was still more pronounced in cultures aerated with a gas mixture containing 15 per cent oxygen, and greatest in the control cultures.

Growth. Growth was most rapid and abundant in cultures aerated with atmospheres containing either 10 or 15 per cent
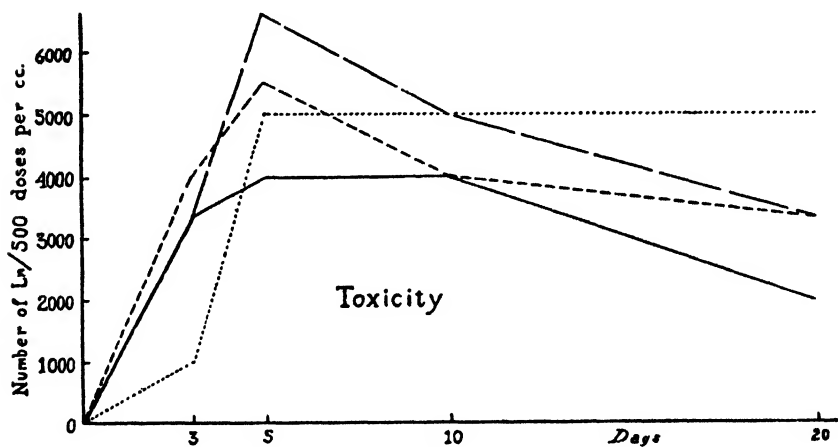
CHART 13. IN THE ABSENCE OF ADDED CO_2

oxygen, and least rapid in cultures grown under a gas mixture containing 5 per cent oxygen.

The pellicles formed on cultures supplied with 5 or 10 per cent oxygen were distinctly membranous in appearance, and were disintegrated with considerably more difficulty than the pellicles formed under ordinary or increased oxygen tensions.

2. In the presence of 5 per cent CO_2. The preceding experiment was repeated except that the gas supply bottles of the three aeration systems contained 5 per cent carbon dioxide; 5, 10 and 15 per cent oxygen were again used. The 4 control cultures were incubated under ordinary atmospheric conditions.

The results are expressed in table 10.

Toxicity (charts 13 and 14). Toxin accumulation was decidedly more rapid and abundant in cultures aerated with test

TABLE 10

Showing the influence of reduced oxygen tension in the presence of 5 per cent carbon dioxide

CULTURE	DAYS INCUBATION	Ln/500 DOSE	pH	NH_3-N IN 100 cc	SEDIMENT IN 100 cc CULTURE	CHARACTER OF GROWTH	VIABLE CELLS PER CUBIC CENTIMETER CULTURE
		cc.		mgm.	cc		
Group I. Controls. Grown under ordinary conditions	3	0.0005	8.6	20 8	0 8	M. C.	
	5	0.0003	8.7	22.7	0 8	M. C.	180,000
	10	0.00025	8 6	17.7	0 7	M. C.	No colony 1/10 plate
	20	0 002	8.7	12.0	0.7	M. C.	No colony 1/10 plate
Group II. Aerated with atmosphere containing 15 per cent oxygen and 5 per cent CO_2	3	0.0002	7 8	26.7	1 2	H. C.	370,000,000
	5	0 00015	8.1	31 8	1.2	H. C.	60,000,000
	10	0.00015	8.1	29.0	1.0	II. C.	23,000,000
	20	0.00015	8.1	32 0	1.1	H. C.	3,000,000
Group III. Aerated with atmosphere containing 10 per cent oxygen and 5 per cent CO_2	3	0.0002	7.8	27 0	1 0	H. C.	310,000,000
	5	0 00015	8.0	30 4	1 1	H. C.	70,000,000
	10	0.00015	8.1	30 7	1 1	H. C.	10,000,000
	20	0.00015	8 1	31 0	1.1	H. C.	5,000,000
Group IV. Aerated with atmosphere containing 5 per cent oxygen and 5 per cent CO_2	3	0 00022	7.8	27 0	0.9	H. C.	210,000,000
	5	0.00015	8.0	30.1	1 0	H. C.	140,000,000
	10	0.00015	8.1	30 7	1.1	H. C.	12,000,000
	20	0 00015	8.1	30 1	1.0	H. C.	No count
Medium			7.5	11.0			

M. C. = moderate crust; H. C. = heavy crust.

atmospheres containing 5 per cent carbon dioxide and either 5, 10 or 15 per cent oxygen than in the control cultures or cultures aerated with CO_2-free atmospheres containing 5, 10 or 15 per cent oxygen.

Maximum potency was attained in all 3 groups of aerated cultures on the fifth day of growth and was maintained throughout the entire twenty-day incubation period.

The most potent culture filtrates obtained during the course of this investigation were obtained from cultures grown under 5 per cent carbon dioxide and either 10 or 15 per cent oxygen.

Growth. Heavy crusts were formed on the surface of all 3 groups of aerated cultures during the first 3 days of growth; however, the greatest amount of bacterial sediment was present
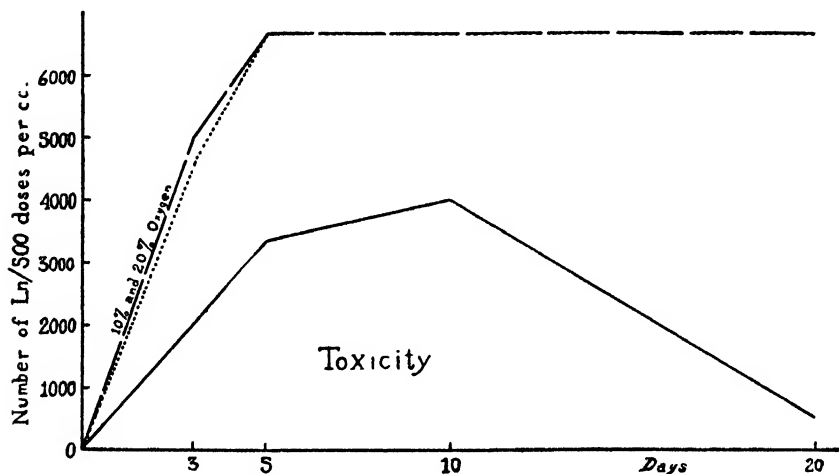
CHART 14. IN THE PRESENCE OF 5 PER CENT CO₂
————— control.
—— —— oxygen tension—15 per cent.
--------- oxygen tension—10 per cent.
.... oxygen tension—5 per cent.

in cultures (shaken) aerated with a gas mixture containing 5 per cent carbon dioxide and 15 per cent oxygen. The number of viable organisms remaining after the period of maximum growth continued at a relatively high figure throughout the twenty-day incubation period.

3. Cultures aerated with an atmosphere containing 1.5 per cent oxygen, with and without added CO₂. In view of the fact that growth and toxin production were but slightly influenced in cultures grown under as little as 5 per cent oxygen, cultures were

aerated with atmospheres containing 1.5 per cent oxygen, with and without added carbon dioxide. Six flasks of broth were inoculated and divided into 3 groups, 2 flasks in each group. Group I was aerated with a CO_2-free atmosphere; Group II was aerated with an atmosphere containing 1.5 per cent oxygen and 5 per cent carbon dioxide and Group III was incubated under ordinary atmospheric conditions, as a control. The results are recorded in table 11.

TABLE 11

Showing the effect of aeration with 1.5 per cent oxygen with and without added carbon dioxide

CULTURE	DAYS INCU-BATION	Ln/500 DOSE	pH	BAC-TERIAL SEDI-MENT IN 100 CC. CUL-TURE	CHARAC-TER OF GROWTH	VIABLE CELLS PER CUBIC CEN-TIMETER OF CULTURE
				cc.		
Group I. Grown under or-dinary atmospheric condi-tions	5	0 0003	8 7	0 9	M. C.	250,000,000
	15	0.0004	9.0	0.9	M. C.	0
Group II. Aerated with atmosphere containing 1.5 per cent O_2 and no CO_2	5	>0 05	7.0	0 15	S. G.	350,000,000
	15	0 0005	7.8	0.5	L. C.	300,000,000
Group III. Aerated with atmosphere containing 1.5 per cent O_2 and 5 per cent CO_2	5	>0 05	6 8	0 10	S. G.	150,000,000
	15	0 002	6.8	0.3	S. G.	230,000,000

M. C. = moderate crust; S. G. = scant growth; L. C. = light membranous crust.

Toxin production and growth were greatly retarded in cultures supplied with as little as 1.5 per cent oxygen; this retardation was greatest in the presence of 5 per cent carbon dioxide. The aerated cultures harvested on the fifth day were practically non-toxic; however, after fifteen days of incubation measurable amounts of toxin were present in both sets of aerated cultures. The fifteen-day cultures aerated with a gas mixture containing 1.5 per cent oxygen and no added carbon dioxide were several times more

potent than the culture subjected to the same amount of oxygen, and 5 per cent carbon dioxide.

Apparently the presence of 5 per cent carbon dioxide in the gas mixture passed over the cultures prevented the usual oxidation of the organic acids which, as shown by Wolf (1922), are formed from amino acids during the initial growth of *C. diphtheriae* in sugar-free peptone broth. As a result the hydrogen ion concentration of the medium remained below pH 7.0 during the 15 day incubation period, thereby inhibiting growth and toxin formation to a greater extent than in the cultures aerated with 1.5 per cent oxygen, without added carbon dioxide.

Remarks. The conclusion may be drawn from the data presented here that the ideal gaseous environment for growth and toxin accumulation is supplied by an atmosphere containing 5 per cent carbon dioxide and 15 per cent oxygen.

4. Cultures grown in the presence of a limited oxygen supply. *C. diphtheriae,* when grown under aerobic conditions on a carbohydrate-free medium, utilizes atmospheric oxygen and gives off carbon dioxide. In order to determine the quantity of oxygen necessary for normal growth and toxin formation, 90 cc. cultures were grown in sealed 3-liter jars with and without added carbon dioxide. In both cases the toxicity of the resulting culture filtrates was greater than the filtrates obtained from the control cultures which were grown under ordinary atmospheric conditions (without seal). Toxin formation was slightly more rapid in the jars containing 3 per cent carbon dioxide at the start than in the jars having ordinary air. The original oxygen content within the sealed jars was reduced from 20 per cent to about 10 per cent, representing a consumption of approximately 300 cc. of oxygen (at 20°C. and 760 mm. pressure) by 90 cc. of broth culture. The carbon dioxide content of the jars containing 3 per cent carbon dioxide at the outset was increased to approximately 10 per cent, while that of the jars originally containing ordinary air was found to be 7 per cent.

That atmospheric oxygen is necessary for toxin formation was shown by growing *C. diphtheriae* in sealed 500 cc. Erlenmeyer flasks containing 90 cc. of medium and ordinary air. Less toxin

was formed under this condition than was formed in the control cultures, and when much larger containers were used. The available supply of oxygen contained in the sealed flasks was practically exhausted.

GENERAL DISCUSSION

The experiments reported herein were undertaken for the purpose of determining the influence of gaseous environment on the growth and toxin production of *C. diphtheriae*, with special reference to carbon dioxide.

From the start, the results indicated that the carbon dioxide tension within the culture flask has a direct bearing on the rate of growth and the amount of toxin produced by *C. diphtheriae*. Aeration of cultures with an atmosphere containing 5 per cent carbon dioxide and from 10 to 20 per cent oxygen resulted in an increased growth momentum, and more rapid and abundant toxin formation, than occurred under ordinary conditions of artificial cultivation. Furthermore, with a given medium, culture filtrates were strikingly uniform in potency. No decrease in toxicity occurred in these aerated cultures on prolonged cultivation at 37°C. While an increased carbon dioxide tension (within the limits mentioned) favored growth and toxin accumulation, greater concentrations than 10 per cent in the atmosphere within the culture flasks increased the hydrogen ion concentration of the medium beyond the optimum for growth and toxin production.

In regard to the oxygen tension necessary for toxin formation, the results have shown that, so long as the concentration of oxygen in the atmosphere within the culture flasks did not fall below 5 per cent, no inhibition of toxin production occurred. As a matter of fact, a slightly reduced oxygen tension appeared to enhance, rather than to retard both growth and toxin accumulation.

The favorable influence of carbon dioxide on growth and toxin formation may depend upon one or more of the following conditions: (1) As shown by Valley and Rettger (1927), carbon dioxide is necessary for the initial growth of *C. diphtheriae*, hence the presence of from 3 to 10 per cent carbon doxide in the atmosphere

within the culture flasks induces early initial cell development. (2) Soon after the maximum growth period sets in, the reaction of the medium in non-carbohydrate cultures grown under aerobic conditions in the absence of added carbon dioxide becomes sufficiently alkaline to be bacteriostatic, if not bactericidal. On the other hand, if the atmosphere in the culture flask contains from 3 to 10 per cent carbon dioxide, the reaction never becomes unfavorable for cell multiplication and toxin accumulation. The importance of preventing the reaction from becoming too alkaline has been demonstrated by Bunker (1919) who was unable to obtain potent toxins in cultures in which the pH value exceeded 8.25.

Similar results have been reported by Dernby and David (1921) who found that the limiting hydrogen ion concentrations for growth were pH 6.0 and pH 8.3. (3) A recent paper by Locke and Main (1928) has indicated that toxin is not accumulated unless there is a definite growth momentum. That growth momentum is increased by an increased carbon dioxide tension has been demonstrated in the present paper. This condition may explain, in part, increased toxin accumulation in cultures aerated with atmospheres containing from 3 to 10 per cent carbon dioxide. (4) An increased carbon dioxide tension prevents the destruction of diphtheria toxin once it is formed. An explanation of the mechanism responsible for the preserving effect of carbon dioxide on diphtheria toxin will be presented in another paper.

The data presented show that the carbon dioxide content of *C. diphtheriae* cultures has considerable influence on growth and on the amount of toxin present at the time of harvesting. This condition, together with the fact that the diphtheria organism produces appreciable quantities of carbon dioxide when grown in sugar-free broth (Wolf, 1922; Apt and Loiseau, 1925), should explain to a considerable degree the lack of uniformity in the toxicity of cultures grown under ordinary atmospheric conditions. That is to say, the rate of destruction of diphtheria toxin varies in different culture flasks containing the same lot of medium, due to the difference in rate at which carbon dioxide

leaves the culture medium after the period of maximum growth. The rate of outward diffusion of the gas from the culture grown under ordinary atmospheric conditions depends upon the character of the medium, size and shape of the culture flask, density of the cotton plugs, and the conditions existing inside the incubator, such as the number of cultures present and the facilities for ventilation.

Dernby and Walbum (1923), have suggested that destruction of toxin in *C. diphtheriae* cultures may be due to the "peptolytic" action of enzymes elaborated by the diphtheria organism. Such an explanation appears to be discredited by the lack of toxin destruction in cultures grown under atmospheres containing from 3 to 10 per cent carbon dioxide.

As will be shown in a following paper, destruction of toxin which usually follows the period of maximum toxicity in cultures grown under aerobic conditions in the absence of added carbon dioxide, is probably due in a large degree to oxidation of the toxin molecule; in the presence of suitable amounts of carbon dioxide this oxidation process is largely or entirely prevented.

SUMMARY

1. The intracutaneous test, as employed in this investigation, was found to be a reliable and economical means of determining the approximate toxicity of *C. diphtheriae* culture-filtrates.

2. Aeration of broth cultures of *C. diphtheriae* with atmospheres containing from 3 to 10 per cent carbon dioxide and from 5 to 50 per cent oxygen resulted in increased growth and toxin production.

3. Aeration of broth cultures with ordinary air or CO_2-free atmospheres containing from 10 to 50 per cent oxygen resulted in marked irregularity in growth and in the toxin content of *C. diphtheriae* cultures. The data obtained show that the higher the oxygen content of the atmospheres passed over the cultures the greater is the irregularity in toxin content of the cultures at the time of harvesting. The rate of destruction of the toxin once it was formed was found to be inversely proportional to the oxygen tension within the culture flask.

4. The optimum oxygen and carbon dioxide tensions for growth and toxin accumulation were found to be supplied by an atmosphere containing from 15 to 20 per cent oxygen and from 5 to 10 per cent carbon dioxide. In the presence of such an atmosphere uniform maximum toxin production occurred in a given medium, and no decrease in toxicity took place on prolonged incubation.

5. The reaction of cultures of *C. diphtheriae* which were grown in meat-infusion broth prepared with Difco-Proteose Peptone was maintained at about pH 8.0, pH 7.8 and pH 7.5 by aeration with atmospheres containing 5, 10 and 20 per cent carbon dioxide, respectively.

6. The ammonia content of all cultures showed a sharp increase during the period of maximum growth. In the case of the cultures grown under ordinary atmospheric conditions, or aerated with CO_2-free atmosphere, this increase was followed by a gradual decline, as compared to a continuous increase throughout the entire twenty-day incubation period in cultures grown under an increased carbon dioxide tension.

7. The amino nitrogen increase in cultures grown in broth prepared with Difco-Bacto Peptone, was much less marked than in cultures grown in broth prepared with Difco-Bacto Peptone, presumably due to a dearth of protein derivatives in Difco-Bacto Peptone which are capable of being broken down into amino acids by the peptolytic enzymes of *C. diphtheriae*.

8. The proteose fractions of certain commercial peptones were found to be necessary for toxin formation for other reasons than their buffering action on decreasing hydrogen ion concentration of the culture medium.

9. Increased carbon dioxide tension was found to prevent the usual rapid destruction of the bacterial cells after the period of maximum growth.

10. The general conclusion may be drawn from the data presented, that carbon dioxide plays an important rôle in the growth and toxin production of *C. diphtheriae,* mainly by:

a. Acting either as a catalyst or food in stimulating growth and toxin formation.

b. Controlling the reaction of the culture medium during growth.

c. Preventing the destruction of the toxin once it is formed, by a mechanism which is as yet little or not at all understood.

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SODIUM CHLORIDE MEDIA FOR THE SEPARATION OF CERTAIN GRAM-POSITIVE COCCI FROM GRAM-NEGATIVE BACILLI

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In culturing mixed clinical specimens, such as urines, in which there are both Gram-positive cocci and Gram-negative bacilli, the difficulty of obtaining pure cultures of the cocci by the usual methods of plating is at times insurmountable. This is especially so when the cocci are present in relatively small numbers, or when the bacilli belong in such genera as Proteus or Pseudomonas. The recent work of one of the authors (White, 1929) showed that colon group organisms could be inhibited or killed by concentrations of urea or of sodium chloride which failed to destroy *Staphylococcus aureus*. This finding suggested the possibility of using such substances in media for the culturing of specimens from cases of mixed infection.

Study of the effect of sodium chloride upon bacteria has been undertaken by many workers. Martens (1888) found that staphylococci were viable on transfer from 30 per cent solution. Petterson (1900), Guillermard (1908, 1909), and others have observed the selective action of salt. So far as we can determine, however, the application of such data for the isolation of salt resistant organisms does not seem to have been considered before. Fischer (1903) classified bacteria in two groups, according to the permeability of their membranes. Among those with permeable membranes, that is, which could not be plasmolyzed, he placed, among others, the Gram-positive spore-formers studied, Proteus, *Esherichia acidilactici*, the sarcinae and the staphylococci. The organisms with impermeable membranes, that is, capable of

undergoing plasmolysis, included the spirilla, *Eberthella typhi*, *Esherichia coli-communis*, *Pseudomonas pyocyanea* and others. Lewandowsky (1904) believed that the action of high percentages of salt upon bacteria was due to the molecular concentrations of the solutions. Holzinger (1908) has shown the inhibitory action of osmosis upon bacteria. The bactericidal action of physiological salt solutions has been demonstrated especially by Duthoit (1923a, 1923b), who found that *Staphylococcus aureus* was the most resistant to physiological salt solution of the organisms studied. In a third paper Duthoit (1923c) showed the retardation of this bactericidal action of sodium chloride by the addition of calcium chloride. Schmidt (1924) transferring to liquid instead of solid media, was unable to confirm Duthoit's results, a difference which may possibly be explained by this variation in method. Neither Duthoit nor Schmidt has considered the reaction of the test solutions.

This report consists of the study of 50 cultures on media containing sodium chloride in concentrations from 1 through 25 per cent. Tests have been made with pure cultures, with cultures mixed in different proportions in the laboratory, and with clinical specimens which showed microscopically the presence of two or more types of organisms.

The following media have been used:

1. Sodium chloride agars. These were beef infusion agars, containing 500 grams of ground beef per liter of water, 1.5 per cent agar, 1 per cent peptone and from 1 through 20 per cent sodium chloride. This medium was adjusted to pH 6.0, tubed, autoclaved and slanted.

2. Sodium chloride broths. These were beef infusion broths, containing 500 grams of ground beef per liter of water, 1 per cent peptone and from 1 through 25 per cent sodium chloride. These broths were adjusted to pH 6.0, placed in flasks in 200 cc. amounts and autoclaved. For the tests, 1 cc. amounts were transferred to small sterile tubes, sterility controls being made at 37.5°C.

Tubes which stood more than twenty-four hours before use were plugged with sterile rubber stoppers, in order to prevent

evaporation, while flasks were kept with their cotton plugs wrapped in tightly fastened rubber.

The hydrogen ion concentration was invariably 6.0 because this had been found by White (1929) to be about the average hydrogen concentration of dog urines in which differential bacteriostasis was first obtained.   It is possible that further work will result in the use of a different hydrogen ion concentration, as other combinations of the variables are studied.

The following cultures have been studied:

	Number of strains
Staphylococcus aureus	5 (nos. 1, 2, 3, 209, 12)
Staphylococcus albus	4 (nos. 5, 6, 8, 11)
Micrococci	5 (nos. 4, 7, 9, 10, 13)
Eberthella typhi	1
Salmonella paratyphi	1
Salmonella schottmulleri	1
Eberthella paradysenteriae Flexner	1
Eberthella dysenteriae Shiga	1
Pseudomonas aeruginosa (B. pyocyaneus)	5
Proteus vulgaris Hauser	4
Klebsiella pneumoniae (B. pneumoniae)	1
Colon group bacilli	18
genus Escherichia	10 (nos. 28, 32, 127, 153, 1, 9, 38, 75, 92, 138)
genus Aerobacter	8 (nos. 25, 89, 170, 73, 90, 96, 190, 8)
Bacillus anthracis	1
Corynebacterium pseudodiphtheriticum	2

TESTS WITH PURE CULTURES

1. Salt agars

Series of slants, prepared as described, and containing from 1 through 20 per cent sodium chloride, were inoculated with 1 standard loopful (5 mm. oise) of an eighteen-hour pH 7.6 broth culture of the test organism.   The effect of the acidity of the medium was controlled by comparison with the growth on a similarly inoculated slant of 1 per cent salt, pH 7.6 agar.   The effect of the salt concentration was controlled by the 1 per cent salt pH 6.0 slant.   Readings were made after twenty-four and forty-eight hours of incubation at 37.5°C.   At the end of this

time, transfers were made to 10 cc. of pH 7.6 broth from slants which showed no visible growth, the surface of the slants being carefully scraped. These transfer tubes were incubated for forty-eight hours. We were therefore able to determine three or four zones of action; first, the salt concentrations which gave no visible inhibition of growth; second, the zone of inhibited but definite growth; third, the zone in which there was no visible growth, but in which there was inhibition without complete killing, as evidenced by positive transfers; and fourth, the zone of salt concentrations in which there was actual killing of the organisms, the transfers being sterile. The results of these tests are recorded in bar diagram I. In this, the solid black bars represent the zones of uninhibited growth, the stripped bars the zones of visible but inhibited growth, and the outlined bars the zones of no visible growth from which positive transfers were obtained. In some cases the bars are not closed, indicating that transfers from the highest salt concentration studied, 20 per cent, were positive. A vertical line closing the outlined bar indicates that transfers from greater concentrations were sterile.

It will be seen from this diagram that there is a sharp differentiation between the cocci and both the Gram-negative and the Gram-positive bacilli. All of the 14 cultures of cocci studied grew heavily through 8 per cent salt, while none of the Gram-negative bacilli grew heavily on higher than 6 per cent salt, some showing inhibition in as low as 4 per cent. Moreover, 3 strains of the cocci grew heavily on 10 per cent salt; 7, or 50 per cent of the strains showed no inhibition on 11 per cent; and 1 strain was uninhibited on 13 per cent. That is, the break between uninhibited and inhibited growth of the cocci lay at about 11 per cent salt, while with the Gram-negative bacilli it came at 5 or 6 per cent. Similarly, the zone of visible but inhibited growth of the cocci lay between 9 and 19 per cent salt as compared with 4 through 8 per cent for the Gram-negative bacilli. The third zone, that of complete inhibition of visible growth, without killing, as shown by positive transfers, began with the cocci at 12 per cent salt. Its upper range was not determined for these organisms, as all of the cocci grew on transfer from 20 per cent
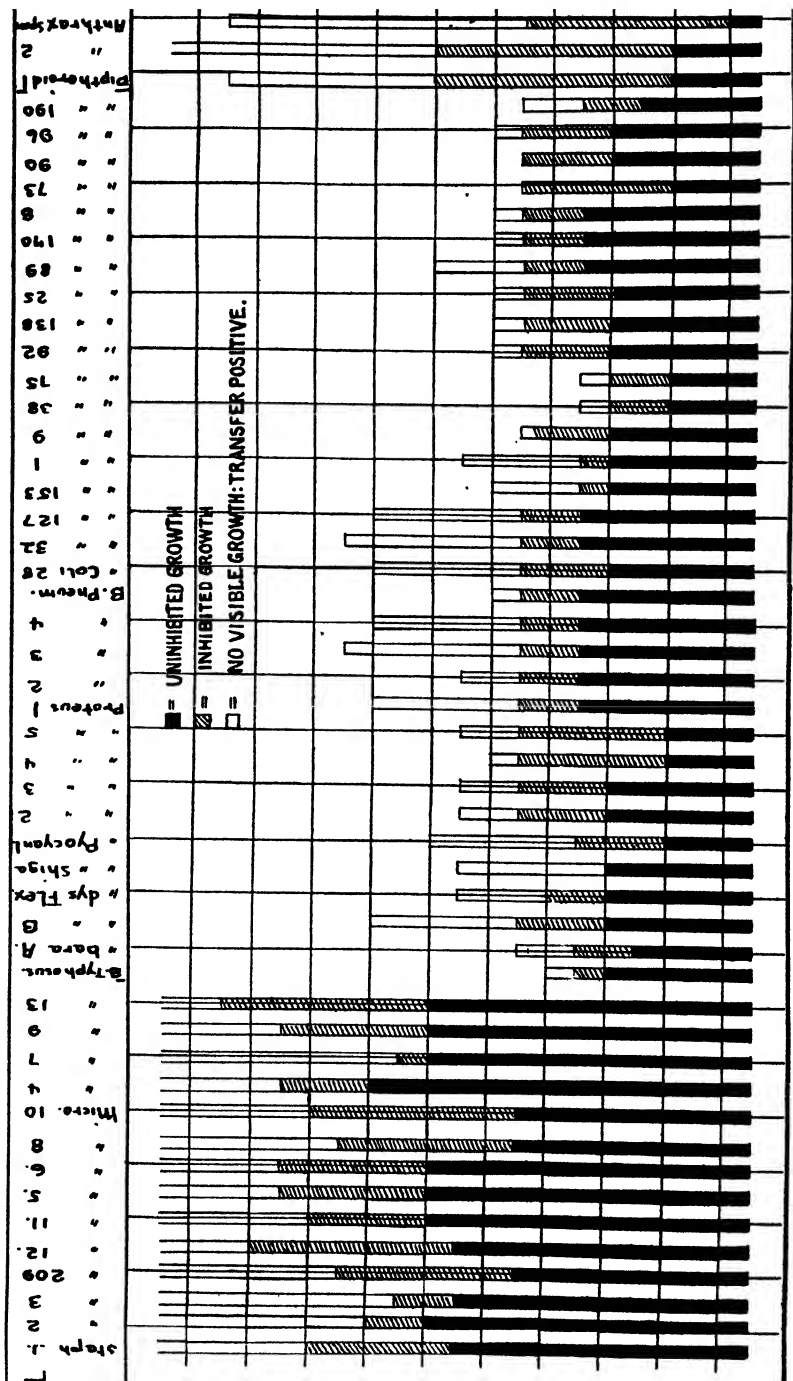
BAR DIAGRAM I. GROWTH OF BACTERIA ON SALT AGARS

salt. With the Gram-negative bacilli, however, this third zone began at 6 per cent and in no case extended beyond 14, all transfers from greater concentrations being sterile. This seems to demonstrate clearly the differential bacteriostasis of sodium chloride, to which the cocci are more resistant than the bacilli.

In regard to the Gram-positive bacilli, it is of interest to note that their growth is inhibited more easily than any of the other organisms studied, the two diphtheroids growing heavily only through 3 per cent salt, the vegetative and sporulating anthrax culture showing inhibition above 1 per cent salt. However, the zone of visible but inhibited growth with all of the cultures of Gram-positive bacilli equals or slightly exceeds the similar zone for the Gram-negative bacilli. The third zone, with the Gram-positive bacilli, is more comparable to that of the cocci, although the vegetative anthrax culture and 1 of the diphtheroids were killed by 19 per cent salt. These results seem to indicate an intermediate position for the Gram-positive bacilli, but it is impossible to draw further conclusions in regard to these organisms until a large number of strains have been studied. Koch, 1881, was the first to show the resistance of anthrax spores to salt, while both de Freytag, 1890, and Stadler, 1899, confirming Koch's statement in regard to spores, found that the vegetative forms of *B. anthracis* were not resistant to salt. By de Freytag's, 1890, method diphtheria bacilli were not killed in three weeks by salt, Stadtler's, 1899, transfers of this organism being positive after four and one-half weeks of salting. Schmidt, 1924, however, found that this organism was soon killed by different salt concentrations in comparison with his results with other organisms. None of these authors, however, so far as can be determined, noted the reaction of their media.

2. Salt broths

One cubic centimeter of broth, prepared as described, was placed in a small tube and inoculated with 1 standard loopful of an eighteen-hour pH 7.6 broth culture of the test organism. Salt concentrations from 1 through 25 per cent were used. The effect of the acidity of the medium was controlled by comparison
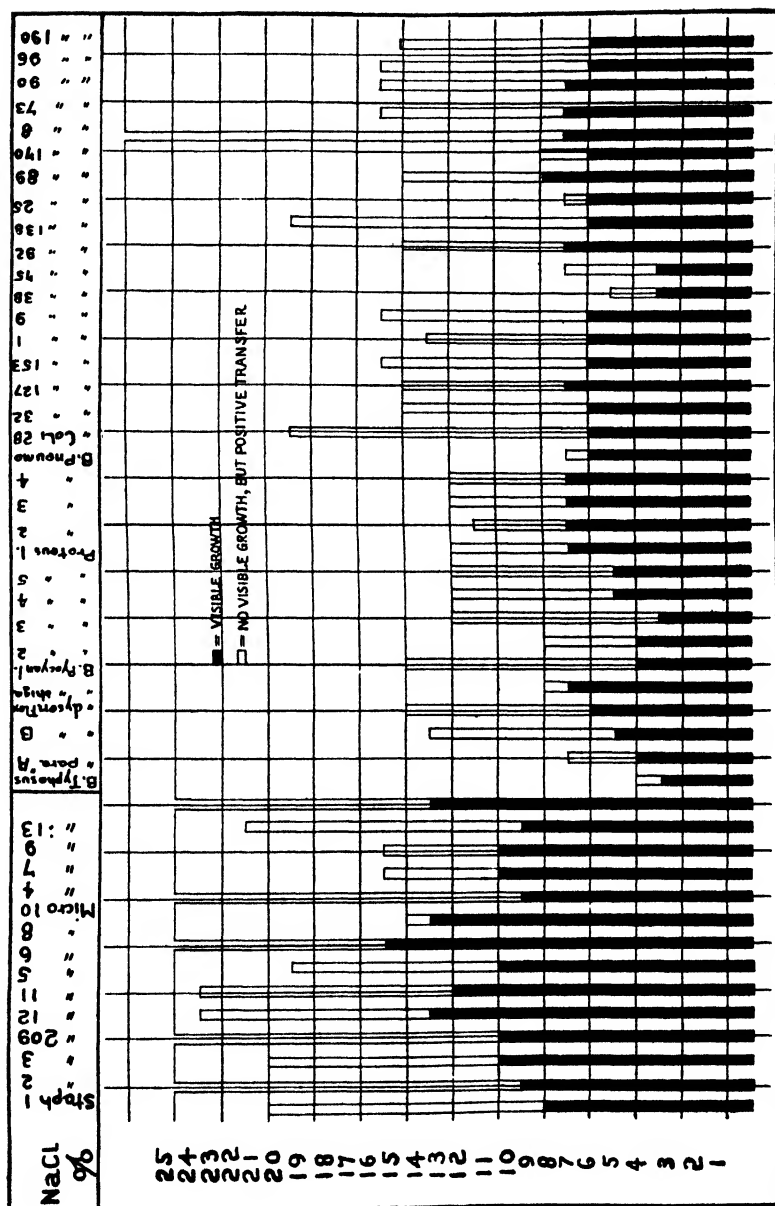
BAR DIAGRAM II. GROWTH OF BACTERIA IN SALT BATHS

with growth in pH 7.6, 1 per cent salt broth, and the effect of the salt by comparison with the 1 per cent, pH 6.0 broth. After twenty-four hours' incubation at 37.5°C., readings were made to determine the presence or absence of visible growth. One-tenth cubic centimeter was then transferred from each tube to 10 cc. of pH 7.6 broth. The transfer tubes were incubated for forty-eight hours at 37.5°C. By these tests it was possible to determine; first, the zone of salt concentration which allowed visible growth; second, the zone of inhibition of growth without complete killing, as shown by positive transfers; and third, in some cases, the zone of complete killing, as shown by sterile transfers. The results of these tests are expressed in bar diagram II.

It is evident that the selective action of sodium chloride may be demonstrated in liquid media, although they are more favorable to the growth of the rapidly developing bacilli. All of the cocci grew well in 9 per cent salt broth, most of them in higher concentrations, 1 strain even in 16 per cent. No visible growth of the bacilli was observed, however, in more than 9 per cent salt. That is, the maximum for the bacilli was the minimum for the cocci. The zone of inhibition of growth without killing showed less striking comparisons between the cocci and bacilli in broth than on agar, but the difference was, in general, still demonstrable. Eighteen cultures, or 53 per cent of the Gram-negative bacilli were killed by the salt concentration of 15 per cent, which was tolerated by the most susceptible of the cocci. The average salt concentration tolerated by the cocci, as shown by positive transfers, was at least 21.7 per cent, while for the Gram-negative bacilli it was 13.6 per cent.

Certain observations may be made in regard to the salt tolerances of the organisms studied. *Eberthella typhi* was the least resistant of all of our cultures. Although de Freytag, 1890, and Stadtler, 1899, by their methods of salting well-developed cultures, obtained positive transfers after long periods of time, other authors, using methods more comparable to ours, have obtained results similar to ours. Thus, Matzuschita, 1900, found the growth of *Eberthella typhi* good on from 0 to 3.5 per cent salt, moderate from 4.5 to 5.5 per cent, scarce on 6.5 per cent and

slight or none above this.   K. von Karaffa-Korbutt, 1912, found that this organism grew in 7 per cent salt in peptone broth, but not in 8 per cent.   Although Schmidt (1924) found *Eberthella typhi* viable in 1.5 per cent salt after ten days, Duthoit (1923a), reported that in 0.9 per cent salt two thousand bacilli were reduced to six within six and one-half hours.   It is possible that a study of a number of strains of this organism and of the related forms will reveal some specific differences.

The effect of salt upon organisms of the genera Pseudomonas and Proteus was striking.   Of the 5 cultures of *Pseudomonas aeruginosa (Bacillus pyocyaneus)* studied, 3 grew heavily on agar only through 3 per cent salt, the other 2 cultures being inhibited above 5 per cent.   These 5 cultures were killed on salt agar by a concentration of not more than 11 per cent salt, as compared with growth on transfer of all the cocci from 20 per cent.   The four Proteus strains behaved alike on salt agar, that is, they all grew heavily on 6 per cent salt, with inhibition through 8 per cent, no transfer being positive above 14 per cent. The findings with both of these genera on salt broth showed a general parallelism, with a somewhat greater tolerance.   The repression of chromogenesis in the Pseudomonas cultures was invariable.   Matzuchita, 1900, also found Proteus more resistant to salt than Pseudomonas, growth of the former being poor above 8.5 per cent, the latter, scarce at 6.5 per cent.

In analyzing the findings with organisms of the colon group and its related form, *Klebsiella pneumoniae,* our lack of adequate classification makes it impossible to draw accurate comparisons. There are no marked differences between cultures of the genus Escherichia and those of the genus Aerobacter. ·These organisms, regardless of their genera, have been studied by India ink examinations for the presence of capsules, in fact some of them were selected from a collection of 200 cultures on account of their encapsulation.   Of the 19 organisms, including the Friedländer bacillus 9, or 47.3 per cent were heavily encapsulated and viscid in growth, 10, or 52.6 per cent showed no capsules, or very slight ones and were not viscid in growth.   Of the 14 cultures which were killed by 9 per cent agar, 9, or 64.2 per cent were the 9

thickly encapsulated cultures. That is, all of the thickly en-
capsulated cultures belonged in the group most easily killed
by salt.

TESTS WITH MIXED CULTURES

The preceding experiments indicated the possibility of using
salt agars to inhibit bacilli present in mixed cultures, or even, in
some instances, to isolate Gram-positive cocci from such mix-
tures of organisms. Eighteen-hour pH 7.6 broth cultures of the
test organisms were mixed in two proportions. Series A con-
sisted of 1 cc. of the culture of a bacillus and 1 cc. of the culture
of a coccus, while Series B consisted of 1 cc. of the bacillus culture
and 1 standard loopful of the coccus culture. One loopful of
such mixtures was placed on each of the following agar slants; 1
per cent salt, pH 6.0 agar, the control and on 6, 8, 10 and 15 per
cent salt, pH 6.0 agars. After forty-eight hours of incubation,
smears of the slants showing visible growth were examined.
Transfers were made to 10 cc. of pH 7.6 broth from the 10 and
15 per cent salt agars, whether or not there was any visible
growth. The use of a liquid medium was favorable to the
development of any bacilli which might be viable.

Enrichment of the cocci was obtained on all of the salt agars
tested. The controls on 1 per cent salt, pH 7.6 agar, on both
series, had heavy growth, either of both organisms, or of an over-
whelming number of bacilli. In the A series of salt agar tests,
in which the inoculum contained equal parts of the bacillus and
of the coccus cultures, examinations of smears of the cultures
showed only cocci in 75 per cent of the tests on 6 per cent salt;
71.4 per cent on 8 per cent salt; 89.2 per cent on 10 per cent salt
and 92.8 per cent on 15 per cent salt. Transfers in this series to
pH 7.6 broth gave pure cultures of the cocci in 17.8 per cent
from 10 per cent salt and in 39.2 per cent from 15 per cent salt.
In the B series of experiments, in which the inoculum was 1
loopful of a mixture of 1 cc. of the bacillus culture and 1 loopful
of the coccus culture, the results on 8, 10 and 15 per cent salt
were fully as good, in some cases better, than in series A. On 6
per cent salt, in series B, 67.6 per cent of the smears showed only

cocci; on 8 per cent salt, the number of smears showing only cocci increased to 91.1 per cent.   On 10 per cent salt, 94.1 per cent of the series B tests showed only cocci, but only 11.6 per cent of the transfers gave pure cultures of cocci.   On 15 per cent salt all of the tests in series B which had any growth, showed only cocci, while of the 27 viable transfers from this concentration, 16 or 59.2 per cent were pure cultures of cocci.

It is of interest to note that the amount of growth on salt agar seems to be determined by the number of cocci in the inoculum.   In the B series, in which there were fewer cocci, the growth was generally lighter, although the number of bacilli was relatively larger than in the A series.   This finding correlates with the results of examinations of smears of the cultures.

TESTS WITH MIXED INFECTIONS

In the application of the previous findings for the enrichment of cocci in mixed infections, the salt concentrations employed must be of sufficiently wide range to cover the unknown variations in the number of organisms present.   In general results with lower concentrations of salt may be obtained from clinical material than from pure or artificially mixed cultures.   This is probably on account of the smaller number of organisms present.

In table 1 are summarized the findings from 20 cases of mixed infection.

The organisms found in these infections were staphylococci, streptococci of the Gamma type, colon group bacilli and *Pseudomonos pyocyanea*, from 2 to 4 types of organisms being present. In 16 of these 20 cases, pure cultures of the cocci were obtained from 6 through 15 per cent salt agar.   Of the 4 cases in which there was enrichment of the cocci, but in which pure cultures were not obtained, 3 were not placed on high salt concentrations. In the fourth case, no. 20, a streptococcus was present in very small numbers with *Ps. pyocyanea* and a colon group bacillus. It seems evident from these 20 cases that the use of salt agars is of definite advantage in culturing many specimens from mixed infections.

TABLE 1

Summary of findings of salt agar cultures of 20 cases of mixed infection

CASE NUMBER	SOURCE	ORGANISMS PRESENT IN DIRECT SMEAR	ORGANISMS OBTAINED BY ROUTINE CULTURE	SALT AGAR CULTURES		
				Salt used	Salt giving mixed culture	Salt giving pure coccus
				per cent	*per cent*	*per cent*
1	Urine	Many bacilli; few cocci	Colon group bacillus; *Staph. albus*	6, 7, 8, 9, 10	7	8 (9 and 10 sterile)
2	Urine	Same as case 1	Same as case 1	6, 7, 8, 9, 10	7	8, 9, 10
3	Wound	Same as case 1	Same as case 1	7, 8, 9	None	7, 8, 9
4	Wound	Same as case 1	Colon group bacillus; *Staph. aureus*	6, 7, 8	6, 7	8
5	Wound	Same as case 1	Colon group bacillus; *Staph. albus*	6, 8, 10, 15	6, 8, 10	15
6	Wound	Same as case 1	Colon group bacillus; *Staph. albus*	5, 6, 7, 8	5, 6, 7, 8	None
7	Urine	Small number of bacilli and cocci	*B. pyocyaneus; Staph. albus*	6, 8, 10, 15	6, 8	10, 15
8	Urine	Small number of bacilli; very few cocci	Colon group bacillus; *B. pyocyaneus; Staph. albus*	6, 7, 8	6, 7	8
9	Urine	Same as case 8	Same as case 8	6, 7, 8	6, 7	8
10	Urine	Many bacilli and cocci	Colon group bacillus; diphtheroid; *Staph. albus*	6, 7, 8, 9, 10, 15	6, 7, 8, 9	10, 15
11	Wound	Same as case 10	Same as case 10	5, 6, 7	5, 6, 7	None
12	Wound	Same as case 10	Same as case 10	6, 7, 8	None	6, 7 (8 sterile)
13	Wound	Same as case 10	Same as case 10	6, 7	6	7
14	Wound	Same as case 10	Same as case 10	6, 7, 8	6, 7, 8	None
15	Wound	Same as case 10	Same as case 10	6, 7, 8	6, 7	8
16	Wound	Same as case 10	Same as case 10	6, 7, 8	6, 7	8

			6, 7, 8	6, 7	8
17	Wound	Same as case 10	Colon group bacillus; B. pyocyaneus; diphtheroid; Staph. albus	6, 7, 8	6, 7	8
18	Urine	Small number of bacilli; many streptococci	Colon group bacillus; Streptococcus (Gamma)	6, 7, 8	None	6, 7, 8 (plain and blood salt agars)
19	Abscess	Many bacilli and streptococci	Colon group bacillus; Streptococcus (Gamma)	6, 7, 8	None	6, 7 (8 sterile)
20	Urine	Many bacilli; few streptococci	Colon group bacillus; B. pyocyaneus; Streptococcus (Gamma)	6, 8, 10, 15	6, 8, 10, 15	None

DISCUSSION

These experiments demonstrate some of the possibilities of the use of salt agars. Should the method prove of value, it may be applied to other organisms than those included here. A study of the use of different salt concentrations in special media for streptococci is indicated from the few cases we have observed. While there is little evidence that salt offers any aid to generic or specific identification, although this was once claimed by Dubois, 1910, for *Escherichia coli*, it is possible that further studies will reveal such differences. We hope that others will test this method or some modification of it on similar or on different types of specimens.

SUMMARY AND CONCLUSIONS

1. It has been found that pH 6.0 sodium chloride agars, with salt concentrations from 2 through 20 per cent, exert marked inhibitory action on the growth of bacilli of the typhoid, paratyphoid, dysentery, and colon groups, on species of Proteus, Pseudomonas, on diphtheroids and on *Bacillus anthracis*. The Gram-positive cocci studied tolerate high salt concentrations, all being positive on transfer from 20 per cent sodium chloride agar.

2. In pH 6.0 broths, with salt concentrations from 2 through 25 per cent, the same differential bacteriostasis may be observed, although to a lesser degree than on agar.

3. It has been found that when mixtures of cocci and bacilli in different proportions are cultured on appropriate salt agars, the cocci invariably outgrow the bacilli and may sometimes be recovered in pure culture.

4. The use of 6, 8, 10 and 15 per cent salt agars greatly facilitates the isolation of Gram-positive cocci from specimens from mixed infections.

5. The use of such salt agars is therefore suggested for the inhibition of Gram-negative bacilli and for the isolation of Gram-positive cocci.

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A CONTINUOUS METHOD OF CULTURING BACTERIA FOR CHEMICAL STUDY[1]

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INTRODUCTION

A continuation of the work done in this laboratory on the chemistry of non-pathogenic bacteria has made it desirable to develop a new technique for growing bacteria in the laboratory in quantities sufficient for chemical examination. An apparatus has been built in which the organisms are grown in a flowing culture medium which is maintained at approximately optimum conditions for growth over a considerable period of time. The apparatus has been found well suited for the cultivation of *Bacterium aerogenes* in synthetic media.

A preliminary chemical study of *Bact. aerogenes* was made in this laboratory by Donald M. Hetler.[3] The ease with which this organism can be grown in synthetic media and its non-pathogenic nature make it well suited for laboratory production. The widespread distribution of the organism in nature, its vigorous fermentive powers, and its common occurrence in the intestinal tract make its chemistry of considerable interest.

DESCRIPTION OF THE APPARATUS

The photograph shows the apparatus with the doors of the incubator open and with the growth tube filled with sterile medium ready for inoculation. The bacteria are grown in the large Pyrex glass cylinder shown inside the incubator.

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[1] This research represents one phase of an extensive investigation dealing with the "Chemistry of Bacteria," which is being carried on by a group of workers in the Sterling Chemistry Laboratory. (Paper No. XXVII)

[2] National Research Council Fellow in Chemistry.

[3] Hetler, Donald M., Jour. Biol. Chem., 1927, lxxii, 573.

HARVEY V. MOYER

PHOTOGRAPH OF THE APPARATUS AS SET UP IN THE LABORATORY

Tracing the culture medium through the system will give a general idea of the plan of construction and of the method of operation. A synthetic culture medium is sterilized in the 25-quart aluminum pressure cooker shown in the upper right of the picture. After sterilization, the medium is allowed to flow through the tube leading from the cooker into the 50-liter Pyrex balloon flask which serves as a reservoir for sterile medium. The
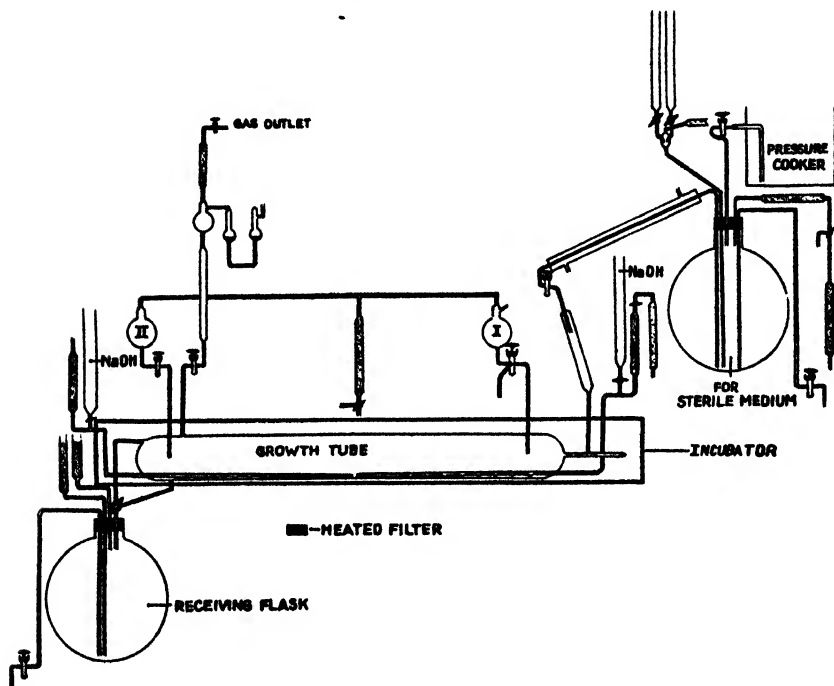
DRAWING OF THE APPARATUS SHOWING THE ESSENTIAL FEATURES

medium is forced from the flask through the coil surrounded by flowing water into a trap which empties into the growth tube through a ½ inch tube which also serves to hold a thermometer. The trap is necessary to prevent the bacteria from growing back into the sterile medium in the 50-liter flask. As fast as the sterile medium is fed into the right end of the cylinder, the medium containing the bacteria in suspension flows out of the

growth tube into the 50-liter receiving flask shown in the lower left of the picture. The medium containing the suspended bacteria can be made to flow from the growth tube into the receiving flask, either through the upper or through the lower tube leading out of the left end of the cylinder. The contents of the receiving flask can be removed when necessary through a tube shown in the diagram. The organisms are separated from the culture medium by use of an electrically driven Sharpless Super-centrifuge and then dried at room temperature in a vacuum oven.

Any gas can be passed through the culture medium by means of aeration tubes which enter each end of the cylinder. Each tube extends to the middle of the large tube and is equipped with jets which permit distribution of the gas throughout the entire length of the body of the culture medium. During the growth of *Bact. aerogenes*, a 10 per cent solution of sodium hydroxide was introduced, as required, into the medium through the aeration tubes. The glass cylinders of about 400 cc. capacity, containing the solution of sodium hydroxide, can be seen in the diagram attached to the external portions of the aeration tubes at each end of the growth tube. The air introduced into the growth tube and the gases formed by the bacteria find an exit through the long vertical tube at the left, marked, "Gas Outlet." A trap filled with concentrated sulfuric acid is sealed to the gas outlet tube in order to relieve sudden changes of pressure in the system. The trap also serves as a pressure gauge.

Effective precautions have been taken in the construction of the apparatus to prevent contaminating organisms from entering the system. All stopcocks are of the mercury seal type. Instead of mercury, however, concentrated sulfuric acid is used as a sealing liquid. The acid makes an effective barrier to the passage of bacteria and is not toxic if small quantities pass into the culture medium. All the air that enters the apparatus is filtered through heated filters, each of which consists of about 8 inches of mineral wool packed tightly in a Pyrex glass tube and kept at a temperature of approximately 250°C. The heating is accomplished by passing an electric current through heating wire which has been wrapped around the filter between layers of

asbestos paper.   In certain places where a rather rapid current of air is passed into the system, the air is filtered through a cotton filter before entering a heated filter.   The end of each outlet tube is kept hot by means of a heated cap, approximately 4 inches in length which slips over the end of the tube.   The 50-liter flasks are stoppered with large rubber stoppers which are partially covered with sealing wax.   During the operation of the apparatus, samples of the culture medium have been removed frequently and have been examined for contaminating organisms. The culture of *Bact. aerogenes* has remained apparently pure throughout several months of operation.

The reaction of the medium in the 50-liter flask on the right can be adjusted through the addition of acid or alkali from the burettes shown in the upper right of the picture.

The incubator is heated by means of two coils of heating wire stretched the entire length of the box under the growth tube. The temperarure is controlled by an automatic electro-thermostat.

OPERATION OF THE APPARATUS

The apparatus has been in operation several months for the mass production of *Bact. aerogenes*.   Synthetic culture media with the following composition are being used:

Medium I

Distilled water.	1000 cc.
$MgSO_4 \cdot 7H_2O$.	0.2 gram
$CaCl_2 \cdot 6H_2O$.	0.01 gram
K_2HPO_4 (crystals).	5.0 grams
NaH_2PO_4 (crystals).	3.3 grams
$(NH_4)_2HPO_4$.	0.6 grams
NaCl.	1.0 gram
Glycerol.	20.0 grams

Medium II

Same as above except that 10 grams of glucose were used instead of the glycerol.

It will be observed that in the above synthetic medium the only source of nitrogen is the diammonium phosphate.   The medium gives a solution with a pH of 6.8 to 7.0 and is used without further adjustment.

A typical operation period in the mass production of *Bact. aerogenes* will illustrate the use of the apparatus. The growth tube was filled with sterile medium and the temperature of the incubator was set at 37°. A suspension of a fecal strain of *Bact. aerogenes* was introduced into the 500 cc. flask marked "I" in the picture. The inoculation was accomplished through the short side arm sealed to the neck of the flask. The tube was opened with a torch and a suspension of the organisms was introduced with a sterile syringe. The side arm was then sealed and the bacteria were allowed to flow past the three-way stop-cock into the right end of the growth tube. The medium was

TABLE 1

Yield of dry bacteria from medium containing glycerol

TIME AFTER INOCULATION	MEDIUM THROUGH THE CENTRIFUGE	DRY BACTERIA
days	*liters*	*grams*
4	32	8.1
6	39	13.9
7	35	12.4
9	45	15.6
11	52	15.0
13	50	13.2
15	36	11.3
16	38	11.1
Total	327	100.6

aerated continuously with sterile air throughout the entire period of the growth. The organisms were allowed to grow in the cylinder for three days before the continuous movement of the culture medium was started. Every twelve hours, 250 cc. samples of the medium were withdrawn from each end of the cylinder and titrated with 10 per cent sodium hydroxide. The calculated quantity of alkali was added to the growth tube through the aeration jets. In this manner the pH of the medium was kept between 6.4 and 7.0. After three days, sterile medium was allowed to flow continuously into the right end of the growth cylinder and the suspension of organisms in the tube was forced out from the left end of the cylinder into the receiving

flask. The data in tables 1 and 2 show the rate of flow and the yield of dry bacteria obtained from each of the modifications of the culture medium.

In twelve hours the reaction of the medium in the right end of the growth tube dropped regularly from pH 7.0 to 6.4, while that in the left end of the tube usually fell to about pH 6.2. From 500 to 600 cc. of 10 per cent alkali were added every twelve hours to bring the medium to pH 7.0.

After the apparatus has been in operation for approximately three weeks, considerable sediment will have collected in the

TABLE 2

Yield of dry bacteria from medium containing glucose

TIME AFTER INOCULATION	MEDIUM THROUGH THE CENTRIFUGE	DRY BACTERIA
days	*liters*	*grams*
2	19	4.7
4	30	14.2
6	32	15.5
8	43	24.2
9	29	18.6
11	35	22.3
13	39	22.5
15	35	24.3
17	34	20.4
Total..................	296	166.7

bottom of the growth tube and in the receiving flask. The growth on the walls of the cylinder will have become extensive and it is advisable to clean the apparatus. The 500 cc. flasks, marked "I" and "II," respectively, are first filled with a suspension of the organisms from the growth tube. The bacteria are kept in the 500 cc. flasks until the growth tube has been cleaned, and serve to inoculate the medium for another period of operation. Cleaning the apparatus can be done very effectively with concentrated hydrochloric acid and steam. After the removal of the medium the acid may be introduced through either one of the aeration tubes. Approximately 2 liters of acid

should be divided between the growth tube and the receiving flask. After the acid has stood in the apparatus for twelve hours, flowing steam should be passed through the system for twenty-four hours. This procedure leaves the cylinder and receiving flask perfectly clean.

DISCUSSION

The apparatus permits excellent control over conditions affecting the growth of the bacteria. The pH of the medium can be kept for an indefinite period of time within the limits most suitable for the rapid multiplication of the organism. The flowing culture medium removes other products of metabolism that would retard growth if allowed to accumulate. The oxygen supply may be controlled quite easily. The apparatus appears to be well suited to the cultivation of anaerobic organisms, although no attempt has been made to grow them.

The multiplication of bacteria in the fresh medium that flows continuously into the growth tube is very rapid because of the high concentration of organisms that are always present in the tube. Most of the bacteria which are thrown out in the centrifuge are probably little more than forty-eight hours old and consequently should have suffered very little extraction of their substance by the culture medium. They come out of the centrifuge almost colorless and have the consistency of soft yeast. When dried and pulverized they form a light gray powder.

There is considerable saving in time and labor over most methods commonly used in culturing bacteria in large quantities in the laboratory. After a little practise, about five hours every two days are required by one person to operate the apparatus.

Because of the size and thickness of the growth tube, the construction of the apparatus offered obvious difficulties in glass blowing, where no special equipment was available for working glass of such size. Two Pyrex glass cylinders 4 feet by 6 inches, open at both ends, were used. The tubes at each end of the growth tube were first sealed into round bottom flasks of the same diameter as the cylinders. The flasks were cut in halves

and the two halves containing the tubes were sealed to the cylinders to form the two ends of the growth tube. The two cylinders were sealed together in the middle by use of an oxygen-hydrogen torch. The writer wishes to express his appreciation of the conscientious work done by the laboratory technician, Mr. F. P. Noble, whose skill in glass blowing made the construction of this special apparatus possible.

In conclusion, the writer wishes to acknowledge the helpful coöperation of Prof. L. F. Rettger who supplied the stock culture of *Bact. aerogenes* and made valuable suggestions concerning the composition of the synthetic culture medium. This work has been done under the general supervision of Prof. Treat B. Johnson whose assistance has been greatly appreciated.

SUMMARY

1. A continuous process for culturing bacteria in the laboratory for chemical study has been described.

2. The construction and operation of the apparatus have been discussed.

3. The mass production of *Bact. aerogenes* has been described.

THE CELL WALL AND THE GRAM REACTION

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The factor or factors upon which the Gram reaction depends have never been satisfactorily determined. Whether the reaction is due to protoplasmic or cell wall differences is a disputed question. Important recent contributions dealing with explanations of the Gram reaction favor the view that the reaction is determined by the protoplasm and that the cell wall is a negligible factor. Stearn and Stearn assume that the chemical nature of the bacterial protein is the determining factor and present some interesting experiments to support their theory that the Gram reaction depends upon the isoelectric point and the pH (Stearn and Stearn, 1923, 1924, 1925, 1928a, 1928b). In attempting to correlate reduction in size in dye exposed anthrax cells with loss of Gram positiveness, Churchman (1927) presents the ingenious theory that the cell consists of an outer layer which is Gram-positive and an inner layer which is Gram-negative. According to this theory, destruction or loss of the outer layer results in the cell becoming Gram-negative. No explanation is offered as to why the outer layer of protoplasm is Gram-positive and the inner layer or medulla Gram-negative.

The strongest evidence in favor of the cell wall as the determining factor in the Gram reaction has been presented by Benians (1912, 1920). His work suggests that the Gram reaction is determined by the physical structure of the cell wall. He excludes the cell protoplasm or its chemical nature as factors in any way affecting the Gram reaction.

The experiments described in the present paper were designed to discover whether the Gram reaction depends upon the cell protoplasm, the cell wall, or both. No attempt was made to

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determine whether the main factor is physical or chemical, though some of the experiments have a bearing on this question. The critical experiments are based largely on the assumption that if the cell wall is not a factor either directly or indirectly in determining the Gram reaction the cell protoplasm should stain the same outside the cell wall as within it. It was realized that if the Gram reaction depends upon the pH of the protoplasm the cell wall might affect the Gram reaction indirectly by interfering with changes in pH. This possibility was taken into consideration in conducting the experiments.

There is some confusion concerning the nature of the cell wall. By cell wall we do not mean ectoplasm but a definite cell or sac. The yeast cell wall is double-contoured and contains a special cellulose resembling metacellulose or fungine. When yeast cells are crushed and the protoplasm extruded, the cell wall retains its entity and appears as an empty case or sac. The cell wall does not stain with the dyes used in the Gram stain. When cells of staphylococci are crushed the empty cell sacs can be seen; and in general appearance they resemble those of yeast cells. The term, cell wall, as used in this article refers to the sac and not to the outer layer of protoplasm.

Since several of the experiments were to deal with extruded protoplasm it was deemed desirable to work with as large a type of cell as possible. For this reason a Gram-positive yeast isolated from a Fleischmann yeast cake was chosen as the main experimental organism. The crucial experiments with this organism were checked by similar experiments with *Staphylococcus aureus* and *B. coli*.

In order to determine whether the factors influencing the Gram reaction in bacterial cells also influence it in yeast cells, a number of experiments were made on the effect of water, acid, alkali and age on the staining of the yeast cells. *Staphylococcus aureus* was used as a control. Burke (1921, 1922) has shown that water added to the decolorizer and treatment of the cells with lactic acid cause the Gram-positive bacteria to become Gram-negative. Elimination of water or treatment with sodium bicarbonate causes such cells to become Gram-positive again. It is a well known fact

that, with age, Gram-positive bacteria tend to become Gram-negative. Experiments similar to those made by Burke demonstrated that water, lactic acid and sodium bicarbonate affect the staining of the yeast cells in the same manner as bacterial cells. The effect was slower, possibly due to the larger size or thicker wall, but was comparable in all other respects. It was also readily demonstrated that the yeast cells in old cultures tend to become Gram-negative, just as do staphylococcus cells. These experiments suggest, but do not prove, that the same factor or factors determine the Gram reaction in yeast cells that determine it in bacterial cells (Burke and Barnes, 1928).

EXPERIMENT 1. TO DETERMINE THE GRAM-STAINING REACTION OF THE EXTRUDED PROTOPLASM OF GRAM-POSITIVE YEAST CELLS

A smear of twenty-four-hour yeast cells was placed between two cover slips and crushed by gentle pressure and a rotary motion. The smears were then stained by the Burke Gram-staining technique.

The broken cells presented an interesting study. The cell wall or sac in some cases was entirely empty, in others there were fragments of protoplasm adhering to the inside. The cracks or fissures through which the protoplasm was forced out were evident in some cases, obscure in others. Surrounding the empty cells were masses of amorphous material. In some cases this could be definitely connected with a cell wall. Unquestionably the amorphous material represented the yeast protoplasm. It was not present on uncrushed smears. Millon's test for protein was positive when applied to crushed yeast cells, negative when applied to uncrushed yeast cells.

The unbroken yeast cells were Gram-positive. The protoplasm outside the cell wall was Gram-negative. The protoplasm remaining in the broken cells in some cases was Gram-positive, in others Gram-negative. This difference in staining of the protoplasm remaining in broken cells may have been due to difference in degree of closure of the fissure through the cell wall after the bulk of the contents was forced out. It could be seen that some

cells closed up more tightly than others.   In no cell in which the protoplasm was Gram-positive could an open fissure be seen.   The fissure was not always evident in cells containing Gram-negative protoplasm.   In all cases the cell wall was unstained.   The results obtained favor the view that the cell wall determines the Gram reaction in the staining of yeast cells.

EXPERIMENT 2.   TO DETERMINE THE EFFECT OF EXTRUSION OF THE PROTOPLASM ON DECOLORIZATION

Duplicate smears of a Gram-positive yeast were prepared and gram stained.   Both smears were Gram-positive.   One smear was now crushed.   Both smears were decolorized and counterstained for the second time.   The uncrushed smear was Gram-positive as in the first case.   The crushed smear consisted of a few intact Gram-positive cells and many broken cells and much amorphous Gram-negative material.   The experiment was repeated with *Staphylococcus albus*.   Similar results were obtained. The experiment was then repeated, with the difference that in order to insure alkalinity and nullify the effect of exposure to the air, 5 per cent sodium bicarbonate was added to the crushed yeast cells before the second application of acetone.   The crushed cells and free protoplasm decolorized as readily as before the application of the alkali.   Acid was not applied because it causes the intact cells to become Gram-negative.

The results obtained suggest that the cell wall determines whether decolorization shall take place.   The following experiments indicate that it does not do this by controlling the pH of the protoplasm but as the result of an impermeability to the dye-iodine precipitate.

EXPERIMENT 3.   TO DETERMINE WHETHER ACID AND ALKALI READILY PENETRATE YEAST CELLS

Smears of yeast cells were prepared and treated for five minutes with brom cresol purple, brom thymol blue and phenol red. The indicator solutions were drained off.   The cells were yellow. A drop of 0.05 per cent NaOH was added.   The cells became

colorless. A drop of ten per cent lactic acid was added. The cells became yellow. A second drop of 0.05 NaOH was added and the cells became colorless. Another drop of the acid, and the yellow color reappeared. Apparently the acid and alkaline solutions used penetrated the cells very readily.

The addition of alkali caused the cells to become colorless. An attempt was made by increasing the time of exposure to the indicators and modifying the strength of, and exposure to, the alkaline solution to cause the cells to take on the red or purple color of the indicators when alkaline. The attempt failed. A faint color was evident to the unaided eye but under the microscope no color was apparent.

Both acid and alkali readily penetrate the cell wall. They also readily penetrate the protoplasm. This applies to the protoplasm outside the cell wall as well as within it. If the protoplasm outside the cell wall is penetrated more readily than that within the cell wall it should be possible to bring about a difference in pH between protoplasm within the cell wall and that outside. And if the Gram reaction is determined by slight differences in pH of the protoplasm it should be possible to make the free protoplasm Gram-positive while the protoplasm within the cell wall remains Gram-negative. Experiment 4 was designed to determine this point.

EXPERIMENT 4. TO DETERMINE WHETHER FREE PROTOPLASM BECOMES GRAM-POSITIVE MORE READILY THAN PROTOPLASM WITHIN THE INTACT CELL

A smear of yeast cells was placed between two cover slips and crushed by gentle pressure and a rotary motion. The smear was made Gram-negative by treatment with acetic acid. A control showed unbroken cells Gram-negative. It was then treated with a solution of sodium bicarbonate and stained by the Gram technique. The intact cells were Gram-positive, the extruded protoplasm Gram-negative.

The results of the experiment suggest that the alkali causes the intact cells to become Gram-positive as the result of affecting the cell wall rather than the protoplasm. They demonstrate that

the protoplasm within intact cells can be made Gram-positive by exposure to alkali more readily than extruded protoplasm.

The possibility of the extruded protoplasm upon exposure to the air and staining solutions becoming more acid while that in the intact cells retains its pH deserves consideration but in view of our results in other experiments we doubt whether this explains the results obtained. The following experiment has a bearing on this question.

EXPERIMENT 5.   TO DETERMINE WHETHER THE EXTRUSION OF THE
PROTOPLASM RESULTS IN A CHANGE IN pH

Smears were made from twenty-four-hour yeast cultures and treated with brom cresol purple, brom thymol blue and phenol red. The cells immediately became yellow, indicating penetration of the indicator and an acid reaction of the protoplasm.

Similar smears were made and the cells crushed as in the previous experiment. The indicator solutions were applied for five minutes. The extruded protoplasm and the protoplasm within the majority of unbroken cells were of the same intensity of yellow. If any difference in pH existed between the extruded protoplasm and the protoplasm within the intact cells the indicators used were not sufficiently delicate to demonstrate it. It is our opinion that if any difference in pH existed it was not sufficient to determine the difference in staining reaction between the extruded protoplasm and the protoplasm within the intact cells.

A few of the intact cells were colorless. Either the indicator did not penetrate, or these cells were more alkaline than the other cells. If these cells were alkaline then the experiment indicated a greater range of pH between the intact cells than between the majority of intact cells and the extruded protoplasm. The same smear was stained by the Gram technique. The intact cells were Gram-positive and the extruded protoplasm Gram-negative. The experiment failed to indicate that the extrusion of protoplasm resulted in a change in pH. Apparently the only difference that existed between the extruded protoplasm and the protoplasm within the intact cells was that the latter were sur-

rounded by a cell wall and the former were not.   All the cells were dead.

EXPERIMENT 6.   TO DETERMINE THE EFFECT OF EQUALIZING THE pH OF EXTRUDED PROTOPLASM AND THE PROTOPLASM OF IN-TACT BACTERIAL CELLS ON THE GRAM REACTION

Smears of *Staphylococcus albus* and *Bacillus megatherium* were prepared and crushed as in preceding experiments.   Intact cells were scattered throughout the masses of amorphous material. The smears were treated with 5 per cent sodium bicarbonate for five minutes and then flooded with the indicators.   The proto-plasm within the intact cells and the extruded protoplasm ap-peared identical under the microscope.   The addition of 10 per cent lactic acid caused all the protoplasm to become yellow. The second application of the sodium bicarbonate caused the pro-toplasm to become colorless again.   With the unaided eye the smears appeared the color of the indicator in its alkaline range but under the microscope the protoplasm all appeared colorless.   The smears were now stained with the Gram technique.   The ex-truded protoplasm was Gram-negative, the unbroken cells Gram-positive.

The results obtained show that in so far as the indicators used are concerned, the bacterial protoplasm outside and inside the bacterial cell may have the same pH and still give a different Gram reaction.   The results suggest that the cell wall and not the pH is the determining factor.   These results agree with those obtained with yeast cells.

We have shown that the protoplasm of dead Gram-positive cells apparently having the same pH may stain differently, de-pending upon whether it is surrounded by a cell wall.   This is true only when the reaction is alkaline or slightly acid.   If ex-tremely acid the protoplasm stains uniformly negative regardless of the cell wall.

The application of indicators to Gram-positive bacteria and yeast cells demonstrated that the protoplasm is normally acid. When the Gram-positive cells received sufficient acid treatment they became Gram-negative (Burke, 1921, 1922).   Subsequent

treatment with alkali caused them to become Gram-positive. When using the minimum amount of alkali and acid that shifts the staining reaction of the Gram-positive bacteria back and forth we find that the staining of the Gram-negative bacteria is unaltered. This applies to dead bacterial cells.   If any difference in pH exists between the Gram-positive and Gram-negative bacteria it is too slight to be detected by the indicators used.

Since the protoplasm of two Gram-positive cells having the same pH may stain differently, depending upon the presence or absence of an unruptured cell wall, it is questionable whether the staining of Gram-negative bacteria having the same pH is determined by the pH rather than by the cell wall.

EXPERIMENT 7.   TO DETERMINE THE EFFECT OF WASHING ON THE pH OF THE CELL AND THE REMOVAL OF INDICATORS

Smears of yeast cells were prepared.   Half of them were washed thoroughly in distilled water.   All were exposed to the indicators. The washed cells were of the same shade of yellow as the unwashed cells.   The distilled water was slightly acid.   Apparently no great change in pH occurred.   The experiment was repeated with slightly alkaline tap water with similar results, the protoplasm remaining acid.

Smears were prepared and treated with the indicators, and then gently washed with distilled water as in the Gram stain.   The indicators were not completely removed, the cells remaining yellow.   The experiment was repeated except that the smears were subjected to prolonged and vigorous washing.   The indicators were completely removed.   The water readily penetrated the cells and removed the indicators unless flowed on very gently for a brief period.   It is conceivable that washing with sufficiently acid or alkaline water might alter the pH of the protoplasm. However, we obtained no evidence that the water as applied in the Gram technique would cause sufficient change in the pH of the protoplasm to affect the Gram reaction.   We doubt whether the difference in pH between tap water and distilled water is sufficient to affect the results in routine Gram staining.

EXPERIMENT 8. TO DETERMINE THE EFFECT OF WATER ON THE pH OF THE DECOLORIZER

A 75 per cent dilution of alcohol was prepared with distilled water. The pH of the water was pH 6.7 and of the 95 and 75 per cent alcohol 6.4. The addition of water to the decolorizer, in some cases at least, fails to cause an appreciable shift in pH. It does, however, increase decolorization of Gram-positive bacteria.

This fact is significant in view of the opposite effect of water and alkali on decolorization. Water favors decolorization of Gram-positive cells, alkali has the opposite effect. Acid added to the cells has the same effect as water. But the action of water on decolorization does not depend upon a change in pH of the decolorizer, certainly not upon a shift to the acid side. The effect of water is the same, irrespective of whether it is slightly acid or slightly more alkaline than the decolorizer or the diluted decolorizer or the protoplasm of the Gram-positive cells. We believe that the action of water in increasing decolorization when added to the decolorizer is independent of its effect on pH. The addition of 2 per cent normal NaOH and 2 per cent normal HCl to the decolorizer shifted the reaction to pH 7.9 and pH 5.8, respectively. The use of these decolorizers did not result in the yeast cells becoming Gram-negative. The addition of 25 per cent water which does not alter the pH of the decolorizer to an equal extent does lead to decolorization. It may do so by affecting the permeability of the cell wall.

We have shown that the addition of water to the decolorizer causes the Gram-positive organism to become Gram-negative. It should also be noted that the drying of the smear just before decolorization favors retention of the dye. Whether this is due to the effect on the cell wall, the pH or the dye is unknown. Possibly this more completely eliminates the action of water on the permeability of the cell wall.

EXPERIMENT 9. TO DETERMINE THE EFFECT OF STRONG KOH SOLUTIONS ON THE GRAM REACTION

Smears of yeast, Staphylococcus and *B. coli* cells were prepared and treated for four hours with the following KOH solutions:

N/1, N/5, N/10, N/20, N/30, N/40, N/50, N/75, N/100.   The cells of the Gram-positive organisms exposed to the stronger alkaline solutions were Gram-negative.   The cells exposed to N/75 KOH were about half Gram-negative.   Those exposed to N/100 KOH were nearly all Gram-positive.   The *B. coli* cells were Gram-negative.

Washing the alkali out with distilled water until the cells were neutral as determined by the use of indicators did not cause those cells that had been rendered Gram-negative to become Gram-positive.   Likewise, neutralizing with HCl failed to cause these cells to stain normally.   When cells made Gram-negative by exposure to N/20 KOH were treated with N/20 HCl and then with 5 per cent sodium bicarbonate they stained normally, the yeast and staphylococcus cells being Gram-positive.   *B. coli* cells similarly treated were Gram-negative.

The results indicate that exposure to a strong alkali does not cause Gram-negative bacteria to become Gram-positive but does cause Gram-positive organisms to become Gram-negative.   This action is reversible in some cases as subsequent neutralization and a weak alkali treatment causes certain cells to stain Gram-positive.   Apparently the N/20 KOH did not produce a permanent change in the cells.

Stearn and Stearn consider the action of the iodine solution in the Gram stain as that of an oxidizing agent and that any other mild oxidizing agent will give satisfactory results in Gram-staining.   The effect of the oxidizing agent, according to these authors is to shift the isoelectric point toward a higher acidity, the acidic strength of a Gram-positive organism thus becoming sufficient to result in retention of the basic dye.   They did not use a counter stain in their experiments.   This omission of one of the steps in the Gram technique is important in considering the bearing of their results on the Gram reaction.   Experiment 10 was designed to determine whether oxidizing agents will serve as mordants in place of iodine and whether the iodine will be as effective if applied before the dye.   If the sole action of the mordant is to oxidize, then it should be effective in influencing the Gram reaction if applied before the dye.

EXPERIMENT 10. TO DETERMINE THE EFFECT OF SPECIAL OXIDIZING AGENTS IN THE PLACE OF IODINE IN THE GRAM TECHNIQUE

A neutralized, normal hydrogen peroxide solution was prepared as recomended by Stearn and Stearn.

Yeast smears were prepared and stained by the Gram technique with the exception that the hydrogen peroxide replaced the Gram iodine. Hydrogen peroxide does not cause a precipitate when added to the dye. The cells were the color of the counter stain. They were now stained with the Gram stain and were Gram-positive. Apparently all oxidizing agents do not serve the same function as the iodine in the Gram technique. Modifying the strength of the hydrogen peroxide and time of exposure did not alter the result.

The hydrogen peroxide was replaced by other oxidizing agents listed by Stearn and Stearn as mordants. Potassium dichromate and potassium permanganate were added to methyl violet solutions. Both caused heavy precipitation. The precipitate caused by the addition of the potassium dichromate to the dye was typical in color and readily soluble in the decolorizer. Potassium dichromate, when used in place of the Gram iodine, caused the yeast cells to be Gram-positive, *B. coli* cells Gram-negative. The precipitate formed as the result of adding potassium permanganate was only slightly soluble in the decolorizer. This precipitate was yellowish brown. When potassium permanganate was used in place of the iodine solution in Gram-staining the cells were yellowish brown and both Gram-positive and Gram-negative cells resisted decolorization. It is obvious that the formation of insoluble precipitates within the cell will result in the cell resisting decolorization regardless of whether it is Gram-positive or Gram-negative.

The results obtained in this experiment indicate that all oxidizing agents will not serve in place of the iodine as mordants in the Gram stain. They suggest that differential Gram-staining requires the formation of a precipitate, independent of protoplasm, upon the addition of the mordant. The precipitate must be soluble in the decolorizer, but not in water. Some oxidizing agents

do not form precipitates when added to the dye and others form precipitates insoluble in the decolorizer. If the Gram reaction depends on the oxidizing effect of the mordant the latter should be effective in influencing the Gram reaction of protoplasm outside the cell wall as well as within it. Other experiments with extruded protoplasm demonstrated that it does not do so.

A series of yeast smears were prepared and flooded with Gram's iodine for five minutes. The removal of the iodine was accomplished in four ways, by draining, by washing in distilled water, by washing with acetone and by washing with an acid water. The four methods were employed because of the possibility of the washing fluid altering the effect of the Gram's iodine. The iodine was washed away because it might prevent the penetration of the dye. The smears were now stained with the Gram technique with the exception that no iodine solution was applied after the dye. The cells were Gram-negative. The oil was washed off with xylene, and the xylene with acetone. The smears were restained, with the iodine applied after the dye. The cells were Gram-positive. We believe that the results obtained favor the view that the iodine affects the Gram reaction by acting as a precipitating agent rather than as an oxidizing agent. Its action is not the same when applied before the dye.

The addition of hydrogen peroxide in place of the iodine solution caused the dye to adhere to the protoplasm, i.e., the cells resisted decolorization. Stearn and Stearn apparently interpreted this as the Gram reaction. They did not apply the counter stain. However, if the counter stain is applied, the cells become the color of the counter stain. This occurs with Gram-negative organisms, Gram-positive organisms and free protoplasm. Replacing the hydrogen peroxide with 5 per cent sodium bicarbonate caused similar smears to resist decolorization to a greater extent. The alkali does not do this by oxidizing or changing the reaction to the acid side. The counter stain readily masked the gentian violet. Treatment of the cells with iodine before the application of the counter stain caused the Gram-negative bacteria and the free protoplasm to decolorize readily upon the application of the decolorizer. The Gram-positive organisms did not decolor-

ize. Hydrogen peroxide and alkali caused the Gram-negative cells and free protoplasm to resist decolorization and iodine had the opposite effect. It is therefore difficult to accept Stearn and Stearn's conclusions that hydrogen peroxide and iodine act in the same manner as regards the Gram reaction, i.e., as mild oxidizing agents.

If we consider the Gram reaction as solely a question of decolorization it follows that organisms that resist decolorization regardless of the cause would have to be called Gram-positive. With the introduction of a counter stain it has become the practice to list as Gram-positive those organisms in which the counter stain does not cover over the gentian violet remaining in the cell. Thus, decolorization is not the sole criterion of Gram-positiveness and as the result of accepted practices the term has taken on a meaning slightly different from its original one. This limits the mordants which can be considered as causing the Gram reaction. Since Gram used iodine-potassium iodide as a mordant we are justified in restricting the term Gram reaction to the reaction produced by this mordant.

The iodine apparently saturates the affinity of the dye and it separates from the protoplasm. A precipitate, insoluble in water, is formed. This is soluble in the decolorizer and readily washes out of the protoplasm and out of the cell unless prevented by the impermeability of the cell wall. The action of iodine in saturating the affinity of another of the triphenylmethane dyes, basic fuchsin, and thus freeing the dye from mucus and protoplasm has been utilized by Burke and Dunning (1924) in a new acid fast stain.

In our opinion the formation of a water-insoluble precipitate is essential to the Gram reaction. Otherwise, the water-soluble counter stain will mix with the gentian violet. Hydrogen peroxide causes the gentian violet to adhere to the protoplasm sufficiently to prevent decolorization but it can never bring about a true Gram reaction because it does not initiate any changes that prevents the mixing of the counter stain and the primary stain. If a drop of gentian violet and a drop of safranin are mixed on a slide the gentian violet color is masked by the safranin. This also

happens if hydrogen peroxide is added before the safranin.   It happens when the gentian violet is adhering to the cell protoplasm.   It does not happen if the Gram iodine solution is added in place of the hydrogen peroxide.   A smear of staphylococci, *B. coli* and yeast cells was stained, treated with hydrogen peroxide and counter stained without decolorization.   All the cells were colored with the counter stain with a trace of gentian violet showing.   A similar smear was prepared and the hydrogen peroxide was replaced with Gram's iodine.   The cells were all Gram-positive.   There was no mixing of the dyes within the cells.   Since no decolorizer was used the *B. coli* cells were not decolorized.

The Gram's iodine in our opinion does three things: reduces the affinity of the dye for the protoplasm, i.e., reduces its staining power, increases the size of the molecules so that the dye is held back in the Gram-positive cells, and makes the dye water-insoluble so that a water-soluble counter stain does not mix with it.

The use of an acid Gram's iodine causes the Gram-positive cells to become Gram-negative.   The action of the acid Gram's iodine resembles that of the neutral Gram's iodine in the saturation of the affinity of the dye for the protoplasm and the formation of a water-insoluble precipitate.   This precipitate washes out of the cells upon the application of the decolorizer.   The effect of the acid Gram's iodine on the Gram reaction may be due to the formation of smaller molecules in the dye-iodine precipitate or to an effect on the permeability of the cell wall.

Another difference between the action of hydrogen peroxide and iodine as regards decolorization may be mentioned.   Drying after the use of Gram's iodine causes the cells to resist decolorization to a greater extent, drying after the use of hydrogen peroxide results in an increase in decolorization.

We have stated that in the Gram-positive cells the dye-iodine precipitate is not masked by the safranin counter-stain, i.e., that there is no mixing of the two dyes.   However, if the exposure to the safranin is prolonged, the cell becomes heavily stained with safranin which masks the gentian violet.   If Gram-positive cells are decolorized for several hours or even over night, they will retain some dye.   With the application of the counter-stain this

is partially masked. Some of the gentian violet comes out with prolonged decolorization. Why it does not all come out at approximately the same rate is unknown. Since this applies only to intact cells the cell wall appears to be the determining factor.

Hydrogen peroxide and alkali, when used in place of iodine, cause the gentian violet to adhere to the protoplasm. This applies to the Gram-negative cells as well as the Gram-positive cells. We have designated this reaction as the pseudo-Gram reaction. Whether the pseudo-Gram reaction ever interferes with the practical application of the Gram stain is doubtful. That it may do so, must be given consideration. Alkali is used in Gram-staining with beneficial results. As applied, any action it may have in causing the Gram-negative organisms to resist decolorization is nullified by the subsequent application of the Gram's iodine. If applied in excess, or if a stronger alkali is used, there is the possibility that its action would not be entirely nullified by the Gram iodine and decolorization might be incomplete. If the counterstain failed to mask the gentian violet remaining in the cells they would appear Gram-amphophile or possibly Gram-positive.

Just how hydrogen peroxide and alkali bring about the pseudo-Gram reaction is unknown. It is not due to any effect on the cell wall because the reaction occurs in free protoplasm.

In discussing the effect of oxidation and the resulting shift toward the acid side, in relation to the Gram reaction and pseudo-Gram reaction, we should consider the effect of the application of acid and alkali. Both acid and alkali readily penetrate the cell. Acid cause the Gram-positive organisms to become acid and Gram-negative. Alkali causes them to become Gram-positive only if the protoplasm is within the intact cell. This applies to cells of a certain age. The effect on the pseudo-Gram reaction is irrespective of the cell wall. As the cells of a Gram-positive organism grow old they tend to become Gram-negative. This is true whether the surrounding medium becomes more alkaline or more acid. *B. subtilis* was placed in peptone water having a pH of 7.2. The cells were Gram-positive at twenty-four hours. At 7 days the reaction was pH 7.9 and fully 50 per cent of the cells were Gram-negative. Staphylococci was placed in lactose broth hav-

ing a pH of 7.2. All were Gram-positive at twenty-four hours. After four weeks they were Gram-negative. The pH of the culture was 5.8. Treatment with alkali brought about the pseudo-Gram reaction. When applied with the Gram stain, only part of the cells reverted to the Gram-positive condition. Staphylococci were placed in peptone water having a reaction of pH 7.2. At the end of four weeks the reaction was pH 7.9 and many of the cells were Gram-negative. In view of our other experiments, we find it more acceptable to explain the changes in the Gram reaction as due to the effect of acid, alkali and old age on the passage of the dye-iodine precipitate through the cell wall rather than on the isoelectric point and the pH of the protoplasm.

EXPERIMENT 11. TO DETERMINE THE EFFECT OF STRONG ALKALI ON THE GRAM REACTION OF B. COLI PROTOPLASM

Smears of a twenty-four-hour culture of *B. coli* were prepared, crushed, exposed for five minutes to 5 per cent sodium bicarbonate and Gram-stained. The cells and extruded protoplasm were Gram-negative. The experiment was repeated with various modifications of the alkali treatment. The time of exposure to the alkali was increased to thirty minutes. Stronger alkali solutions, such as normal NaOH and N/20 NaOH were applied to the smear and drained off at the termination of the period of exposure, to the iodine solution, and to the dye on the smear. In no case did the cells or the extruded protoplasm become Gram-positive. Treatment with acid likewise failed to cause these cells to become Gram-positive. The use of indicators demonstrated that the alkali caused the protoplasm, both inside and outside the cell wall, to become alkaline. We do not know what degree of alkalinity was attained but apparently the protoplasm was saturated with the alkali. The alkaline solution was drained off, not washed off. Apparently the protoplasm was as alkaline as it could be made with the solutions used.

We have shown that similar alkali treatment renewed the Gram reaction of intact yeast cells and of Gram-positive bacteria made Gram-negative as the result of acid treatment and, since extruded protoplasm was not so effected, it is suggested that this

was due to the effect on the cell wall rather than on the proto-plasm. Apparently the differences in staining between Gram-positive and Gram-negative bacteria can not be due wholly to differences in pH of the protoplasm.

It is true that with alkali treatment the extruded protoplasm and that within the intact cells of *B. coli* shows a slight retention of the dye. Apparently the effect of the alkali was not entirely nullified by the Gram's iodine. This is evident if an examination is made before the counter-stain is applied. Upon application of the counter-stain this is no longer evident. We believe that Stearn and Stearn rated similar reactions as Gram-positive reactions. We do not, however, consider such retention of the dye by the protoplasm as comparable to the Gram reaction as commonly known and utilized by bacteriologists but as an entirely different phenomenon, determined by the alkali treat-ment. Similarly the extruded protoplasm of Gram-positive yeast cells can be made to retain some of the dye by strong alkali treatment but this is readily covered over by the counter stain and is not comparable to the staining within the cell wall.

According to Benian the physical character of the cell wall determines the Gram reaction, i.e., the cell wall of the Gram-positive bacteria does not readily permit the molecules of the dye-iodine precipitate to pass through with the decolorizer. It follows that if the dye-iodine precipitate is easily washed out of the Gram-negative cell by the decolorizer it should be possible to reverse the process and get the dye back into the cell. This should be true provided the treatment does not alter the cell wall, causing it to become impermeable.

EXPERIMENT 12. TO DETERMINE THE PERMEABILITY TO DYE-IODINE PRECIPITATE OF THE CELL WALL OF A GRAM-POSITIVE ORGANISM MADE GRAM-NEGATIVE BY THE ADDITION OF ACID

A dye-iodine precipitate was formed by adding Gram's iodine to a 1 per cent methyl violet. The precipitate was washed and, without drying, a saturated solution in acetone was prepared. Yeast smears were treated with N/20 HCl for twenty-four hours.

Such treatment causes the cells to become Gram-negative. Other smears were crushed and the balance left intact as controls. The smears were covered with the dye-iodine-acetone solution. This dried quickly, in about the time required to decolorize such cells in the Gram technique. Upon examination, no difference could be noted between the cells treated with acid and those untreated. Those treated with acid were now Gram-stained and found to be Gram-negative. Surrounding the cells and adhering to the cell wall in some cases were dye granules. These were round or oval and about the size of large cocci. In no case could it be demonstrated that the dye had passed inward through the cell wall. Apparently it did not pass inward and come out again as the acetone evaporated. The extruded protoplasm in crushed smears was unstained. When a dye-acetone solution was added to similar smears the dye penetrated the cells and protoplasm. The cell wall of both the Gram-negative and Gram-positive yeast cells held back the dye-iodine molecules but not those of the iodine free dye.

The results obtained failed to demonstrate that the cell wall of Gram-positive yeast cells made Gram-negative by acid is more permeable to the inward passage of the dye-iodine solution than normal cells. Possibly some uncontrolled physical factor prevented the inward movement of the solution into the cell. While these results fail to add strength to the cell wall theory of the Gram reaction they also fail to confirm the chemical theory because according to this theory the cell wall has nothing to do with the reaction, and therefore the dye-iodine solution should pass with equal readiness through the wall of both the Gram-positive and Gram-negative cells.

The cell wall theory assumes a change in the size of the dye molecules with the addition of the iodine or mordant. According to Stearn and Stearn the addition of the iodine can not increase the size of the molecules sufficiently to check the passage through the cell wall. This experiment suggests that the iodine, in some way, alters the dye so that it passes inward through the cell wall less readily. To this extent the results of this experiment favor the cell wall theory of the Gram reaction.

DISCUSSION

Our main experiments described in this paper support Benian's experiments and add weight to some of his conclusions. We do not see how our results can be explained on any basis except that of the cell wall theory of the Gram reaction. Stearn and Stearn have presented much data in support of their conception of the isoelectric point and the pH as the determining factors in the Gram reaction.

Thus the evidence bearing on the Gram reaction is conflicting and we must search for further data that will bring all the evidence into harmonious support of one theory or the other or we must prepare a new theory that accounts for all the known facts.

It is our belief that the conflict of evidence is due to a confusion of two phenomena, a confusion between the true Gram reaction and a pseudo-Gram reaction. This second reaction, apparently, is the reaction with which Stearn and Stearn are mainly concerned and to which some of their experimental evidence applies. This is a reaction evidenced by the union of dye and protoplasm. If, as a working basis, we accept these two phenomena then the conflict of evidence concerning the nature of the Gram reaction in part at least disappears, because it can readily be seen that much of the experimental evidence considered as bearing on the chemical nature of the Gram reaction in reality has no bearing on this reaction.

We must point out, that any one reviewing the evidence should bear in mind that any data concerning live cells may not apply because no one knows what the Gram reaction of any living cell is. Gram-staining kills the cells. Permeability of the cell wall changes with death. Furthermore, some of the results obtained without the use of a counter-stain should have questionable weight because, as we shall explain later, one can not always tell whether a cell is Gram-positive or Gram-negative unless the counter-stain is applied. Likewise, the staining results obtained without a precipitating mordant should be viewed with suspicion as possibly not bearing on the true Gram reaction. Viewed in this light the evidence against the cell wall theory of the Gram reaction is weakened and possibly eliminated.

Our conception of the pseudo-Gram reaction already referred to is that it results from a union of dye and protoplasm. The cell wall takes no part in this reaction. The reaction occurs readily inside or outside the cell wall. The reaction does not result in the protoplasm being heavily stained. It is so stained that when a counter stain is applied the primary stain is largely masked and the protoplasm said to be Gram-negative. This explains why we should view critically any staining results obtained without the use of a counter-stain. Without the counter-stain the cell might wrongly be classed as Gram-positive.

The possibility of the pseudo-Gram reaction occurring at the same time and affecting the Gram reaction deserves comment. Our experiments indicate that the Gram's iodine by saturating the affinity of the gentian violet for the protoplasm eliminates the pseudo-Gram reaction in the Gram-negative organisms and free protoplasm. Presumably the same thing occurs in the Gram-positive organisms but, since these resist decolorization after the Gram's iodine is applied, it is difficult to determine what has happened within the cell. The results obtained with the staining of the free protoplasm of Gram-positive cells favors the view that the two reactions do not occur at the same time. According to our conception of the two reactions it is theoretically impossible for this to happen. It is conceivable, however, that an incomplete Gram reaction may be superimposed on a partially eliminated pseudo-Gram reaction. A weak iodine solution or a strong alkali solution may possibly cause this to happen. While alkali properly applied favors both the pseudo-Gram reaction and the Gram reaction we do not believe that it favors the Gram reaction indirectly by its action on the pseudo-Gram reaction. We believe that it affects the passage of the dye-iodine precipitate through the cell wall.

We accept the cell wall theory of the Gram reaction. Protoplasm outside this cell wall strictly speaking can not be made Gram-positive. The gentian violet either is washed out by the decolorizer or mixes with the counter-stain. A Gram-positive cell can be made Gram-negative by breaking the cell wall. Normal differences in the pH or isoelectric point of the protoplasm

do not account for differences in Gram reaction. Gram-positive cells can also be made Gram-negative by adding water to the decolorizer. The Gram reaction is also affected by the use of an alkali. Acid treatment causes the cells to become Gram-negative. Subsequent alkali treatment causes these cells to become Gram-positive only when the cell wall is unbroken. This demonstrates that the cell wall is a factor, and that it is not a factor as a result of preventing the penetration of the alkali because there is no hindrance to the penetration of the alkali through the rupture of a broken cell or into free protoplasm and yet these remain Gram-negative. Any factor that affects the passage of the dye-iodine precipitate through the cell wall affects the Gram reaction. Consequently altering the size of the molecule of the dye-iodine precipitate or altering the permeability of the cell wall will affect the Gram reaction.

We reject the chemical or protoplasmic theory of the Gram reaction. This theory does not explain our experimental results and requires certain assumptions which are in some cases difficult to accept. We frequently find a chain of streptococci with the middle cell Gram-negative and all the other cells Gram-positive. The protoplasmic theory of Stearn and Stearn requires the assumption that the protoplasm of this cell differs in pH or in isoelectric point from that of the cells on each side and from which it arose. All the cells have been killed and fixed by heat. Since all can be treated with acid and alkali without changing the result we doubt whether the protoplasm of the single cell is sufficiently more acid to account for the difference in staining. We hesitate to accept the necessary assumptions as to differences between the protoplasm in the cells in view of the fact that all that is necessary to cause the other cells to become Gram-negative is to break the cell walls. Breaking the cell wall after the death of the cell may conceivably affect the pH of the protoplasm but we question whether any other change occurs. Subsequent acid and alkali should level the pH of the protoplasm of the broken cell with that of the neighboring cells. Our experiments indicate that this happens and that the cells continue to stain differently. If the extrusion of the protoplasm does result in a change in pH and thus

accounts for the change in staining, we must still consider the cell wall a factor indirectly affecting the staining by affecting the pH.

If there are, as we suggest, two phenomena confused in the discussion of the Gram reaction we must conclude that certain factors affect both phenomena.   The alkali affects both the Gram reaction and the pseudo-Gram reaction.   And we are forced to the further conclusion that alkali affects the Gram reaction by its action on the cell wall or the dye-iodine precipitate and that it affects the pseudo-Gram reaction in some other manner.

In our experiments in which the cells were first stained and then crushed and the protoplasm forced out, we failed to find any evidence in support of the conception of Churchman that in Gram-positive organisms the protoplasm consists of an outer Gram-positive layer and a Gram-negative center.   The extruded protoplasm appeared uniformly stained.   We worked with yeast cells and Churchman worked with anthrax which may account for our failure to find support for his conception.   Churchman has made the suggestion that the mechanism of the Gram reaction is not necessarily the same in the case of bacteria of different species reacting in an identical manner to the Gram stain.   The relation between the irregular staining of the anthrax protoplasm observed by Churchman and the true Gram reaction is obscure.

The Gram-positive organisms are in general more susceptible to the antiseptic action of gentian violet than Gram-negative organisms and attempts have been made to show that Gram-positiveness and susceptibility are parallel and due to the same factor. It is our opinion that the Gram reaction depends on the cell wall. Sensitiveness to the antiseptic action of the dye may be associated with the phenomenon we have called the pseudo-Gram reaction in which the dye in water soluble form and with antiseptic action unimpaired unites with the protoplasm.   In the Gram reaction the dye is in insoluble form and has probably lost its antiseptic action.   If susceptiblity of the cell to the bactericidal action of gentian violet is bound up with the pseudo-Gram reaction this requires that the pseudo-Gram reaction and the Gram reaction be largely parallel because susceptibility and the Gram reaction are largely parallel.   There are exceptions to this parallelism.

To what degree the pseudo-Gram reaction and the Gram reaction are parallel remains to be determined. Gram-positive cells are surrounded by a more imperious cell wall as indicated by the Gram reaction. This phenomenon appears to be parallel with the greater susceptibility of the protoplasm to the dyes but whether it accounts for it is problematical.

CONCLUSIONS

1. The Gram reaction of yeast cells is due to, and influenced by, the same factors that determine and influence the Gram reaction of bacterial cells.

2. The bacterial cell has a definite cell wall. After the cell is crushed and the protoplasm extruded the empty sac can be seen. In appearance it resembles the empty sac of a crushed yeast cell. Both are unstained after exposure to the Gram stain.

3. The breaking of the cell wall causes the protoplasm of Gram-positive bacteria, and of yeast cells, to become Gram-negative. The protoplasm within intact cells and the extruded protoplasm may have the same pH and stain differently. The extruded protoplasm can not be made Gram-positive.

4. Acid and alkali readily penetrate the cell wall. They interfere with the Gram reaction by affecting the passage of the dye-iodine precipitate through the cell wall. They alter the affinity between dye and protoplasm but this is not part of the Gram reaction though it may interfere with it to a minor extent. Acid and alkali, as well as water, probably affect the Gram reaction by altering the permeability of the cell wall.

5. The functon of the mordant in the Gram technique is that of a precipitating agent. The precipitate must be insoluble in water, soluble in the decolorizer. It must less readily pass through the cell wall of the Gram-positive organisms. The precipitation of the dye results in its losing its affinity for the protoplasm.

6. The affinity of the aqueous gentian violet for both Gram-negative and Gram-positive bacteria or the extruded protoplasm can be increased by hydrogen peroxide and alkali. Protoplasm so treated resists decolorization. This reaction has been confused with the true Gram reaction. It is nullified by the action

of Gram's iodine.   We have designated this reaction as the pseudo-Gram reaction.   Since hydrogen peroxide and alkali are quite different agents it is possible that more than one reaction is involved in what we have termed the pseudo-Gram reaction.

7. Any factor that alters the cell wall or the dye-iodine-precipitate may affect the Gram reaction.   The protoplasm of the cell plays no part in the Gram reaction.   Bacteria are Gram-positive, Gram-negative or Gram-variable depending upon the permeability of the cell wall.

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A RAPID METHOD FOR OBTAINING THE VOGES-PROSKAUER REACTION

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It is a well known fact that in the "V-P" reaction there must elapse a period of from thirty minutes to twenty-four hours before the color becomes perceptible, and that constant shaking and an increase in temperature hasten the reaction.

A review of the literature upon the subject indicated that the slow development of color was very probably the result of the slow oxidation of acetyl-methyl-carbinol, resulting from the cleavage of glucose, to diacetyl, the latter being the compound which enters into combination with some nitrogenous degradation product of peptone and so causes color formation. The rate of color formation being dependent therefore upon the rate of availability of the reactive substances, it occurred to the writer that a mild oxidizing agent would, under appropriate conditions, effect a more rapid oxidation of the acetyl-methyl-carbinol and so hasten the appearance of the colour.

Experiments were therefore carried out with sodium peroxide, which under the following conditions gave the appropriate "V-P" color reaction in two minutes or sooner.

The culture medium consisted of proteose-peptone, glucose, and dipotassium hydrogen phosphate, which was inoculated with a known positive test organism, and incubated at 37° for a definite period of time. To 2.5 cc. of the culture medium, 10 mgm. of sodium peroxide were added and immediately afterwards 1 cc. of 40 per cent sodium hydroxide; the culture tube was then placed in boiling water for one minute and then vigorously shaken. In less than one minute the color became perceptible.

[1] Holder of the T. Eaton Company Scholarship.

For the positive tests the organisms *Aerobacter aerogenes* and a strain of *Bacillus mucosus-capsulatus* (Klebsiella group) were used, while the negative test was demonstrated with cultures of *Escherichia communior*, *Proteus vulgaris*, and *Alcaligenes fecalis*.

In this test positive results were obtained occasionally with a twenty-four-hour culture but the most intense color was obtained with seventy-two-hour cultures. It is of course obvious that acetyl-methyl-carbinol formation is a function of bacterial activity and the duration of culturing must be conditioned by that factor.

ADAPTATION OF THE METHOD TO LABORATORY PRACTICE

Instead of weighing out 10 mgm. sodium peroxide, sufficient can be held on the end of the wire of an inoculating needle, the loop of which has been bent to the shape of the letter "M," or any other solid surface will do just as well. This is then placed in the culture medium and gently warmed over the Bunsen flame, at the same time constantly agitating the contents of the tube.

The addition of too great a quantity of the sodium peroxide will cause further oxidation of the diacetyl compound and consequent loss of color. Any doubt as to the positivity of the test can be set at rest by pouring the contents of the culture tube into a white porcelain evaporating dish; the white background makes a more definite contrast and any colour can be readily detected. This has been found a convenient adjunct in the laboratories here.

OBSERVATIONS ON LUMINOUS BACTERIA

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Inoculations of luminous bacteria (*Bacillus Fischeri*, Beijerinck, Migula) (Migula, 1900) on the surface of agar slants of phosphate buffered medium were made by one of us (Shoup, 1928) and sealed in pure hydrogen. These cultures were found to grow and regain luminescence on re-exposure to the air after two months. Preparations made in the same manner have now been found to regain growth and luminescence after fourteen months in the absence of oxygen. It has also been found that no growth or luminescence occurs in the presence of pure nitrogen, but that luminous bacteria sealed in pure nitrogen regain growth and luminescence after twelve months.

Inoculations were also made on agar slants of the calcium carbonate buffered medium previously described (Hill, 1928) and sealed without displacing the air. These were kept at 3.5°. Faint luminescence and slow growth continued for six days until all of the oxygen was consumed. In those tubes opened within five months luminescence reappeared at once and the transfers appeared normal. After twelve months most of the bacteria were dead as shown by the reappearance of luminescence only after the tube had been open to the air for about twenty-four hours, and by the development of only scattered colonies on the transfers. On the other hand, transfers from inoculations on the phosphate buffered medium appeared quite normal after twelve months, even when these had been sealed without displacing the air.

It has been stated (Hill, 1928) that luminous bacteria were soon killed in borate buffers above pH 9.0, the assumption being made that the buffers were non-toxic, but it was later found that

these bacteria were unharmed in NaOH solutions at pH 9.0 (Hill, 1929), and that they survive for about thirty minutes at pH 10.0.   Luminous bacteria grow and luminesce best on alkaline media.   In the earlier paper, the poor luminescence and slow growth on the barium carbonate buffered medium (pH 9.2) was ascribed to the too-great alkalinity of the medium.   It now appears probable that a specific toxic action of the barium ion was also concerned.   Bacteria have been grown on a medium buffered with magnesium carbonate and magnesium hydroxide, both of which are strongly alkaline (in the biological range), the latter slightly more alkaline than the barium carbonate medium.   On both the magnesium carbonate and magnesium hydroxide media the light was very brilliant and growth was good, continuing with full brilliance for twenty days, and lasting with lesser brilliance for twelve days longer.   While the magnesium hydroxide medium was initially the more alkaline, both reached the same pH after a few days, as judged by thymol blue in the medium.

If bacteria are suspended in sea water and shaken with the solid buffers employed, the light is fully brilliant after one hour in calcium and magnesium carbonates, is faint in barium carbonate, and is extinct in magnesium hydroxide.   After three hours it is extinct in barium carbonate also.   In other words, luminous bacteria live and grow vigorously on the surface of medium having a reaction which is fatal if they are immersed in it.   It is suggested that this is because of the constant formation of acid by the bacteria and its diffusion into the solid medium, protecting them from direct contact with the excess alkalinity, while in suspension this protection is lost through rapid mixing of the solutions, due doubtless in part to the motility of the organisms, (in spite of their motility, spreading on the surface of medium if the proper salinity does not occur).   If this hypothesis is correct, a simple explanation is offered for the death of the bacteria in the course of months without growth on the surface of a medium initially favorable (calcium carbonate buffered), and their continued viability on the surface of a medium slightly less favorable for growth, but of lower reaction.

In table 1 are given the pH values for sea water shaken with the indicated solids, then the pH after boiling, then, after three hours aeration. The drop in pH of sea water shaken with calcium carbonate is explained by McClendon (1917, 1918) as due to the precipitation of the excess calcium carbonate from the supersaturated solution normal to sea water. Atkins (1922) in a discussion of the substances responsible for the reaction of sea water, gives some figures for the reaction produced by boiling MgO with water (pH 10.0), for $MgCO_3$ boiled with $MgCl_2$(pH 9.2), and for $CaCO_3$ boiled with $CaSO_4$ (pH 8.0); and these are

TABLE 1

	1	2	3	4
Sea water	8 4	8 48	8.02	
Sea water + $CaCO_3$.	7 67	8 55	8 14	7 6-8 0
Sea water + $BaCO_3$ . .	8 94	9 06	9 21	9.2
Sea water + $MgCO_3$ . . .	9 13	8 93	9 02	9 0
Sea water + $Mg(OH)_2$ ...	9.92	9.41	9 31	9 4

1. pH (with glass electrode) of sea water, shaken with substances indicated.
2. Same, after boiling, cooling under tap, and measuring at once.
3. Same, after three hours aeration.
4. pH (colorimetric) of culture media (1 per cent glycerol, 1.5 per cent peptone, 1 5 per cent agar, 1 per cent beef extract, added to sea water-buffer mixtures, and autoclaved fifteen minutes at 15 pounds).

probably as close as could be expected to the reactions of the same substances boiled with sea water.

The precipitation of Ca and Mg from sea water has been discussed by Irving (1926), by Haas (1922), and by Kapp (1928).

From the figures of Irving and of Haas it seems likely that practically no magnesium was precipitated by calcium and barium carbonates even when boiled with sea water, and that very little calcium was precipitated by either barium or magnesium carbonates. There may have been some precipitation of calcium by the magnesium hydroxide, perhaps 20 per cent (Irving). Kapp unfortunately does not give the pH values of her solutions, so it is hard to apply them here. The reaction of sea water boiled with excess solid should depend principally on the partic-

ular solid added, and should approach very closely that of the culture medium buffered with the same substance. This is found to be the case, as may be seen by comparison of the values given in the third and fourth columns of the table.

Luminous bacteria were inoculated into plain broth, broth containing 1 per cent glycerol, and broth containing 1 per cent glucose. Samples were tested at intervals for several days for indications of the presence of indol by Ehrlich's test (Boehme, 1906). No instances of color reaction were noted, indicating that indol was absent. This species of luminous bacteria is in no sense putrefactive.

Stab cultures into nutrient gelatine of pH 8.0 show abundant growth at the surface after twenty-four hours, and liquefaction begins after forty-eight hours, proceeding down the entire length of the inoculation to form a crater of liquefaction, reaching maximum depth in four days. Luminescence occurs at the top of the crater, occurring in the depths only after complete liquefaction of the stab. A flocculent precipitate gradually collects at the bottom of the crater. Stab inoculations in nutrient gelatine with 4 or 5 cm. of gelatine poured in above will show luminescence at the top of the inoculation and finally growth and liquefaction along the stab.

Luminous bacteria inoculated into plain broth made up in sea water and adjusted to pH 8.0 cause no change in the reaction of the medium, but if 1 per cent glycerol is present the pH will fall to 7.0 in twenty-four hours, without gas production. If 1 per cent glucose is present the culture becomes highly acid within twenty-four hours with marked decrease of luminescence.

If they are grown on agar slants without glycerol there is again no evidence of acid production, and growth and luminescence are both very poor.

SUMMARY

Preservation of luminous bacteria (*B. Fischeri*) in presence of pure hydrogen and pure nitrogen is discussed, also their preservation in absence of oxygen on two kinds of media, with an explanation of the difference in result. Reaction of culture

media produced by excess of different solid buffer substances is discussed. Barium carbonate, alone, of the substances used is toxic, while magnesium carbonate is apparently stimulating. These luminous bacteria do not produce indol. They liquefy gelatine. They produce acid from glycerol and glucose, but luminesce very little on glucose.

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STUDIES ON CARBON DIOXIDE

V. THE MECHANISM RESPONSIBLE FOR THE PRESERVING ACTION OF CARBON DIOXIDE ON DIPHTHERIA TOXIN[1]

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INTRODUCTION

In two previous publications (1927 and 1929) the writers stated, as a result of rather extensive observations, that carbon dioxide within certain limits of concentration stimulates growth and toxin production of *C. diphtheriae*, and that it acts as a preservative of the toxin after it is formed. The mechanism responsible for this preserving action of carbon dioxide constitutes the subject matter of the present paper.

It has been generally observed that under the usual conditions of preparation of diphtheria toxin a more or less rapid decrease in the potency of the toxin occurs soon after the stage of maximum toxicity is reached. Several theories have been advanced regarding the toxin deterioration. According to these suggestions, the decrease in toxicity may be due (1) to oxidation of the toxin, (2) the influence of increased OH ion concentration, or (3), as Dernby and Walbum (1923) and Dernby (1923 and 1926) have suggested, the action of a peptolytic enzyme elaborated by the diphtheria organism itself during the process of growth.

As will be shown later, the first two of these theories probably explain, at least in part, the detoxifying process which takes place during prolonged cultivation under ordinary atmospheric conditions. The explanation offered by Dernby seems highly improbable, in view of the fact that in cultures grown under

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[1] This paper embodies part of the work presented in the senior author's Doctorate Thesis deposited in the Yale University Library.

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atmospheres containing from 10 to 20 per cent oxygen and from 3 to 10 per cent carbon dioxide maximum toxin formation occurred in the adopted medium, and no decrease in toxicity took place on long-continued incubation (Plastridge and Rettger, 1929).

METHODS

Toxicity determinations. The intracutaneous test described in a previous paper (1929) was employed. Briefly, the method is as follows:

Dilutions of the toxin solution were prepared in such a way that 0.05 cc. of the resulting solution contained the exact amount of toxin to be injected. At the same time a solution of antitoxin containing $\frac{1}{25}$ unit[2] per cubic centimeter was prepared. Equal portions of the diluted toxin and antitoxin solutions were mixed and allowed to stand for fifteen minutes. Exactly 0.1 cc. of the toxin-antitoxin mixture was then injected intradermally into the shaven skin of a white guinea pig. The smallest amount of toxin which, when injected together with $\frac{1}{500}$ unit of antitoxin, produced a small area of necrosis distinguishable five days after the injection, was recorded as the Ln/500 dose. The results in the tables which follow are expressed in Ln/500 doses.

Aeration. An aeration system similar to the one described in the previous paper (1929) was employed in the experiments in which the test gas was bubbled through the toxin solution.

EXPERIMENTAL

I. The influence of molecular oxygen on diphtheria toxin

Diphtheria toxin has been considered by most investigators as easily alterable by oxidizing agents. That cultures of *C. diphtheriae* decrease in toxicity on standing was first observed by Roux and Yersin (1889). Brieger and Boer (1896) observed that in a weakly alkaline solution toxin was affected by the oxidizing agent, potassium permanganate, to a much greater extent than by the reducing substance, ferrous sulphate. Sieber (1901) found that diphtheria toxin is rapidly destroyed by

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[2] One unit of antitoxin in this case is defined as the amount of antitoxin which will exactly neutralize 1 M.L.D. of diphtheria toxin.

calcium peroxide. Ozone was added to the list of destructive oxidizing agents by Arloing and Troude (1903).

De Potter (1923) repeated some of the previous work mentioned and concluded that, while diphtheria toxin may be destroyed by the peroxides of hydrogen, potassium and calcium, and by potassium permanganate and ozone, it is not as easily oxidized as is generally believed. A slow current of air passed over a culture filtrate of *C. diphtheriae* for a period of forty-eight hours did not cause any appreciable loss in toxicity.

TABLE 1

Showing the effect of molecular oxygen on the toxicity of filtrates from cultures grown under ordinary atmospheric conditions

NUMBER OF HOURS AERATION	GAS PASSED THROUGH SAMPLE	DIRECT EXPOSURE (BY BUBBLING) TO CO_2-FREE ATMOSPHERE CONTAINING 50 PER CENT OXYGEN		DIRECT EXPOSURE (BY BUBBLING) TO ATMOSPHERE CONTAINING 50 PER CENT OXYGEN AND 5 PER CENT CARBON DIOXIDE	
		Ln/500 dose	pH	Ln/500 dose	pH
	liters	*cc.*		*cc.*	
0	0	0.0002	9 0	0.0002	9 0
1	2	0 00025	9 2	0 0002	8 0
3	6	0 0003	9 1	0.0002	8.0
8	16	0 0006	9.1		
24	40	0.001	9 1	0 0002	8.0
48	90	0 002	9 1	0.0002	8.0
72	120	0.004	9.1	0 0002	8.0

A. Preliminary tests. *a.* A strongly toxic filtrate from a culture of *C. diphtheriae* which was *grown under ordinary atmospheric conditions* was selected for the first test.

An atmosphere containing 50 per cent oxygen was passed through a 20 per cent solution of sodium hydroxide, to remove traces of carbon dioxide, and then bubbled through a 30-cc. portion of the culture filtrate. One cubic centimeter of aerated material was removed from time to time, deposited in a small test tube and packed in ice until the toxicity tests were made (never longer than twenty-four hours). The hydrogen ion concentration of the aerated culture filtrate was determined each time that a 1 cc. sample was removed.

The above procedure was then repeated on another portion of

the same lot of culture filtrate, but with the use of an atmosphere containing 50 per cent oxygen and 5 per cent carbon dioxide. The results are recorded in table 1.

b. A similar experiment was conducted on a portion of filtrate from a culture of *C. diphtheriae* which was grown under an atmosphere containing 5 per cent carbon dioxide and 20 per cent oxygen. The results are expressed in table 2.

The preliminary tests showed that diphtheria toxin was not destroyed by bubbling an atmosphere containing 5 per cent carbon dioxide and 50 per cent oxygen through cell-free culture

TABLE 2

Showing the effect of molecular oxygen on the toxicity of filtrates from cultures grown under an increased CO_2 tension

NUMBER OF HOURS AERATION	GAS PASSED THROUGH SAMPLE	DIRECT EXPOSURE (BY BUBBLING) TO CO_2-FREE ATMOSPHERE CONTAINING 50 PER CENT OXYGEN		DIRECT EXPOSURE (BY BUBBLING) TO ATMOSPHERE CONTAINING 50 PER CENT OXYGEN AND 5 PER CENT CARBON DIOXIDE	
		Ln/500 dose	pH	Ln/500 dose	pH
	liters	*cc.*		*cc.*	
0	0	0.00015	8.0	0.00015	8.0
1	2	0 00015	8 7		
2	4	0 002	8.8		
8	16	0 00025	8.8	0 00015	8 0
24	48	0.0004	9.0	0.00015	8 0
48	90	0.0008	9 0	0.00015	8.0
78	130	0 002	9.0	0.00015	8 0

filtrates of *C. diphtheriae* for a period of seventy-two hours, even though the temperature of the aerated material was 37°C. On the other hand, a marked decrease in toxicity occurred in portions of the same lots of filtrate which were aerated with a CO_2-free atmosphere containing 50 per cent oxygen.

The hydrogen ion concentration of filtrates aerated with a gas mixture containing 5 per cent carbon dioxide was maintained at pH 8.0, while the hydrogen ion concentration of filtrates aerated with CO_2-free atmosphere remained at or near pH 9.0 during the aeration process.

A decrease in hydrogen ion concentration from pH 8.0 to pH 9.0 occurred in filtrates from cultures grown under increased CO_2

tension, upon aeration with a CO_2-free atmosphere, due to the removal of carbon dioxide. It is of interest to note that decrease in toxicity of these filtrates is retarded during the first few hours of aeration, or until the carbon dioxide removable by aeration is complete.

B. The influence of hydrogen-ion concentration on the rate of destruction of diphtheria toxin by molecular oxygen. The possibility that the difference in hydrogen ion concentration may account for the difference in the rate of destruction of diphtheria toxin in filtrates aerated with a CO_2-free gas mixture and those aerated with a gas mixture containing 5 per cent carbon dioxide was considered.

A stock supply of highly toxic filtrate was prepared, placed in a tightly stoppered flask and stored in the refrigerator. Previous to making a test, a 60-cc. portion of the stock material was removed and adjusted to the desired hydrogen ion concentration by the addition of several drops of normal sodium hydroxide or normal lactic acid, as the case required.

Two tests were conducted on portions of the filtrates which had been adjusted to a given reaction. In the first test a CO_2-free gas mixture containing 50 per cent oxygen was bubbled through the 30-cc. portion of toxic material under test, at the rate of 2 liters per hour. The second test was performed in a similar manner on the duplicate 30 cc. sample, with the exception that the test atmosphere contained 5 per cent carbon dioxide along with the 50 per cent oxygen.

To determine whether a decrease in toxicity was directly due to the hydrogen ion concentration of the aerated material or to the action of molecular oxygen on the toxin, a 1 cc. sample was removed from the test portion of filtrate before aeration was begun, and stored at 37°C. in a tightly stoppered test tube until the completion of the aeration process (96 hours). The 1 cc. sample was then removed and its toxin content determined. The results are recorded in table 3 and expressed graphically in chart 1.

Filtrate adjusted to pH 6.0. A rapid decrease in toxin content occurred in filtrates adjusted to pH 6.0 and aerated with either a CO_2-free atmosphere containing 50 per cent oxygen, or one

TABLE 3

Showing the effect of molecular oxygen and hydrogen ion concentration on the rate of destruction of diphtheria toxin

NUMBER OF HOURS AERATION	VOLUME OF GAS PASSED THROUGH SAMPLE	pH 6.0 CO₂-free Ln/500 dose	pH 6.0 CO₂-free pH	pH 6.0 5 per cent CO₂ Ln/500 dose	pH 6.0 5 per cent CO₂ pH	pH 7.0 CO₂-free Ln/500 dose	pH 7.0 CO₂-free pH	pH 7.0 5 per cent CO₂ Ln/500 dose	pH 7.0 5 per cent CO₂ pH	pH 8.0 CO₂-free Ln/500 dose	pH 8.0 CO₂-free pH	pH 8.0 5 per cent CO₂ Ln/500 dose	pH 8.0 5 per cent CO₂ pH	pH 9.0 CO₂-free Ln/500 dose	pH 9.0 CO₂-free pH	pH 9.0 5 per cent CO₂ Ln/500 dose	pH 9.0 5 per cent CO₂ pH
	liters	*cc.*		*cc.*		*cc.*		*cc.*		*cc.*		*cc.*		*cc.*		*cc.*	
0	0	0.0002	6.0	0.0002	6.0	0.0002	7.0	0.0002	7.0	0.0002	8.0	0.0002	9.0	0.0002	9.0	0.0002	9.0
1	2					0.0002	7.0	0.0002	7.0					0.00025	9.0	0.0002	8.0
4	8	0.0004	6.1	0.0004	6.0	0.0002	7.1	0.0002	7.0	0.0002	8.0	0.0002	8.0	0.0003	9.2	0.0002	8.0
8	16													0.0006	9.1	0.0002	8.0
24	48	0.0006	6.1	0.0004	6.0	0.00025	7.1	0.0002	7.0	0.0003	8.0	0.0002	8.0	0.001	9.1	0.0002	8.0
48	96					0.0003	7.1	0.0002	7.0	0.0006	8.0	0.0002	8.0	0.002	9.1	0.0002	8.0
72	140	0.002	6.1	0.002	6.0	0.0004	7.1	0.0002	7.0			0.0002	8.0	0.004	9.1	0.0002	8.0
96	180	0.005	6.1	0.003	6.0	0.0005	7.1	0.0002	7.0	0.001	8.0	0.0002	8.0	0.006	9.1	0.0002	8.0
Sample kept at 37°C. not aerated		0.002	6.0			0.0002	7.0			0.0002	8.0			0.00025	9.0		

Note: Filtrates under the heading CO₂-free were aerated with a CO₂-free atmosphere containing 50 per cent oxygen. Filtrates under the heading 5 per cent CO₂ were aerated with an atmosphere containing 50 per cent oxygen and 5 per cent CO₂.

containing 50 per cent oxygen and 5 per cent carbon dioxide. A similar decline in toxicity occurred in the sample of filtrate which was adjusted to pH 6.0 and stored at 37°C. but not aerated.

Evidently, destruction of diphtheria toxin adjusted to a reaction of pH 6.0 is primarily due to the effect of hydrogen ions on the toxin, rather than to an oxidative process.

Filtrate adjusted to pH 7.0. A gradual decrease in toxicity occurred in filtrates adjusted to pH 7.0 and aerated with a
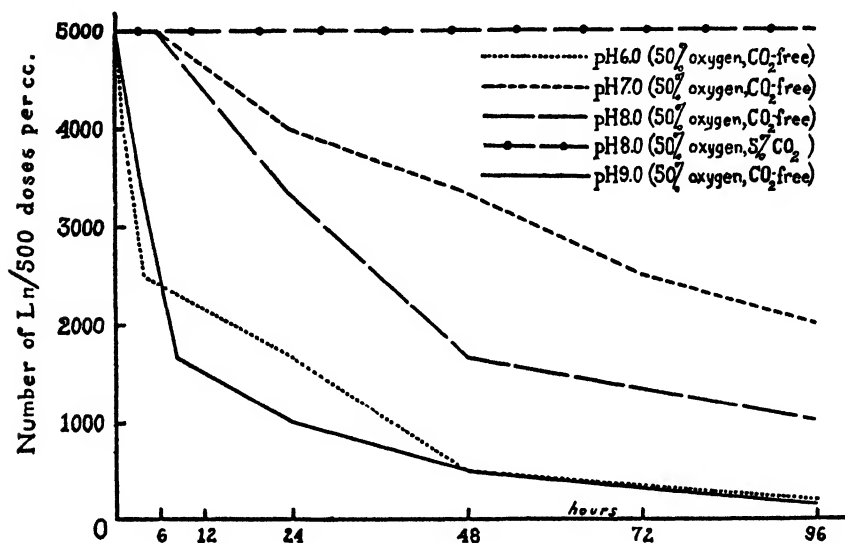

The legend shown on the chart:
- pH6.0 (50% oxygen, CO_2-free)
- pH7.0 (50% oxygen, CO_2-free)
- pH8.0 (50% oxygen, CO_2-free)
- pH8.0 (50% oxygen, 5% CO_2)
- pH9.0 (50% oxygen, CO_2-free)

CHART 1. THE EFFECT OF H-ION ON THE DETOXIFICATION OF DIPHTHERIA TOXIN BY OXYGEN

CO_2-free atmosphere containing 50 per cent oxygen. The rate of toxin destruction was less rapid than in filtrates adjusted to any other reaction.

No loss in potency occurred in filtrates which were adjusted to pH 7.0 and aerated for ninety-six hours with 50 per cent oxygen and 5 per cent carbon dioxide. At the same time, no loss in toxicity occurred in the sample kept in a tightly stoppered test tube at 37°C. for a period of ninety-six hours.

Filtrate adjusted to pH 8.0. The rate of decrease in toxicity was more marked in filtrates adjusted to pH 8.0 and aerated with

a CO_2-free gas mixture containing 50 per cent oxygen than in filtrates adjusted to pH 7.0. However, the rate of decrease was not as rapid as in filtrates adjusted to pH 9.0.

Again, no loss in toxicity occurred either in samples kept at 37°C. in stoppered tubes, or in the filtrates adjusted to pH 8.0 and aerated with 50 per cent oxygen and 5 per cent carbon dioxide.

Filtrates adjusted to pH 9.0. A sharp decrease in toxicity occurred during the first eight hours of aeration in filtrates adjusted to pH 9.0 and aerated with a CO_2-free atmosphere containing 50 per cent oxygen. The decrease in toxicity was more gradual during the remainder of the 96-hour aeration period.

A slight decrease in toxicity occurred in the 1 cc. sample kept at 37°C. in a stoppered tube for a period of 96 hours. The destruction of toxin under this condition, however, was not at all comparable to the pronounced destruction which took place in the material aerated with the CO_2-free atmosphere.

The hydrogen ion concentration of the 30 cc. portion of filtrate which was aerated with 50 per cent oxygen and 5 per cent carbon dioxide increased to pH 8.0 where it remained during the aeration process. As would be expected, no decrease in toxicity took place in the test material which was aerated with the test gas mixture containing 5 per cent carbon dioxide.

Discussion. The results obtained show that the lower the hydrogen ion concentration (within the limits of pH 7.0 and 9.0) the more rapid the destruction of diphtheria toxin by molecular oxygen. A similar observation was made on the hemotoxin of the Welch bacillus by Neill (1926), who found that within the range of from pH 6.0 to 9.0 the rate of oxidation of the hemolysin is roughly proportional to the alkalinity of the toxin solution. The rate of oxidation of blood has also been shown to be related to hydrogen ion concentration by Mathison (1911) and Kato (1915). They found that small amounts of alkali hasten the rate of oxidation of blood, while an acid reaction retards oxidation and favors reduction.

The fact that CO_2 prevented the destruction of diphtheria toxin in filtrates adjusted to a hydrogen ion concentration between pH 7.0 and pH 8.0 and then aerated with an atmosphere

containing 50 per cent oxygen and 5 per cent carbon dioxide, whereas destruction did occur in filtrates having the same reaction which were aerated with a CO_2-free atmosphere, shows that carbon dioxide may prevent the destruction of diphtheria toxin in other ways than by regulating the reaction of the toxin solution.

A similar study made on purified toxin would undoubtedly yield more striking results, as it is very probable that non-toxic substances in a culture filtrate of *C. diphtheriae*, such as proteoses and peptones, may exert a buffering action against the oxidation of the toxin. Nelis (1926) observed that purified diphtheria toxin was more easily destroyed by ozone, formol, sodium oleate and the salts of quinine than was unpurified toxin. Koulikoff and Smirnoff (1927) found that phosphate buffer solutions (pH 8.0) of purified diphtheria toxin decreased more rapidly that did unpurified toxin.

Attempts to reactivate solutions of diphtheria toxin which had been partially detoxified by the action of molecular oxygen were made, employing sodium hydrosulfite as the reducing agent. Under the conditions of the experiment none of the partially detoxified toxin filtrates regained any of their original toxic properties which had been lost during treatment with molecular oxygen.

II. The influence of hydrogen ion concentration and gaseous environment on the stability of diphtheria toxin

Different lots of diphtheria toxin are known to vary considerably in respect to stability, even when stored under identical conditions. In view of the preceding experiments, it seems highly probable that the carbon dioxide content and the hydrogen ion concentration of different lots of toxin may explain to a considerable degree this difference in rate of deterioration. Accordingly, tests were conducted for the purpose of determining the effect of carbon dioxide gas and the reaction of the toxin solution on the rate of deterioration of the toxin in filtrates stored under carbon dioxide, nitrogen and air over long periods of time.

Three different lots of culture-filtrates designated as A, B and

TABLE 4

Showing the influence of hydrogen ion concentration and CO_2 on the stability of diphtheria toxin

Temperature of storage 37°C.

CONDITIONS OF STORAGE	LOT OF TOXIN	AT START		10 DAYS		30 DAYS		70 DAYS		100 DAYS		180 DAYS	
		Ln/500 dose	pH	Ln/500 dose	pH	Ln/500 dose	pH	Ln/500 dose	pH	Ln/500 dose	pH	Ln/500 dose	pH
Under CO_2	A	0.0005	8.0	0.0005	6.9	0.0008	7.0	0.0008	6.9			0.001	6.9
	B*	0.0003	8.6	0.0003	6.8	0.0003	6.8	0.001	6.8	0.0003	6.9	0.008	6.8
	C	0.00015	9.0	0.00015	6.9	0.0002	6.9						
Under N	B	0.0003	8.5	0.0005	8.5	0.0008	8.3	>0.01	8.3			>0.01	8.3
	C	0.00015	7.0	0.0002	7.0	0.0003	7.0			0.0005	7.0		
Filtrates adjusted to various hydrogen ion concentrations and stored in sealed tubes containing approximately 10 cc. of air and 5 cc. of filtrate	A	0.0005	8.8	0.004	8.8	>0.01	8.8	>0.01	8.8			>0.01	8.8
	B	0.0003	9.0	0.02	9.0	>0.03	9.0	>0.05	9.0			>0.05	9.0
	C	0.00015	9.0	0.001	9.0	0.0015	9.0			0.006	9.0		
	B	0.0003	8.5	0.0005	8.5	0.0008	8.5	>0.01	8.5			>0.05	8.5
	C	0.00015	8.5	0.0006	8.5	0.0012	8.5			0.003	8.5		
	B	0.0003	8.0	0.0004	7.8	0.0008	7.8	>0.03	7.8			>0.05	7.8
	C	0.00015	8.0	0.0003	8.0	0.001	8.0			0.002	8.0		
	A	0.0005	7.0	0.002	7.2	0.003	7.2	0.004	7.4			>0.01	7.4
	B	0.0003	7.0	0.0005	7.0	0.0008	7.0	>0.01	7.0			>0.01	7.0
	C	0.00015	7.0	0.0003	7.0	0.0008	7.0			0.0008	7.0		
	B	0.0003	6.0	0.0015	6.0	0.05	6.0	>0.05	6.0			>0.05	6.0
	C	0.00015	6.0	0.005	6.0	0.01	6.0			>0.05	6.0	>0.05	6.0

* It seems probable that sufficient CO_2 to exert a deteriorating effect on the toxin, due to too great hydrogen ion concentration, was present in these portions of filtrate.

> No reaction was produced by injecting the amounts of filtrate preceded by this symbol.

C were employed in the following test.[3]   Six 5-cc. portions of A
were stored under carbon dioxide gas.   Similar portions of fil-
trates B and C were stored under carbon dioxide gas and under
nitrogen.   At the same time samples of B and C were adjusted
to pH 9.0, pH 8.5, pH 8.0, pH 7.0, and pH 6.0 and stored in
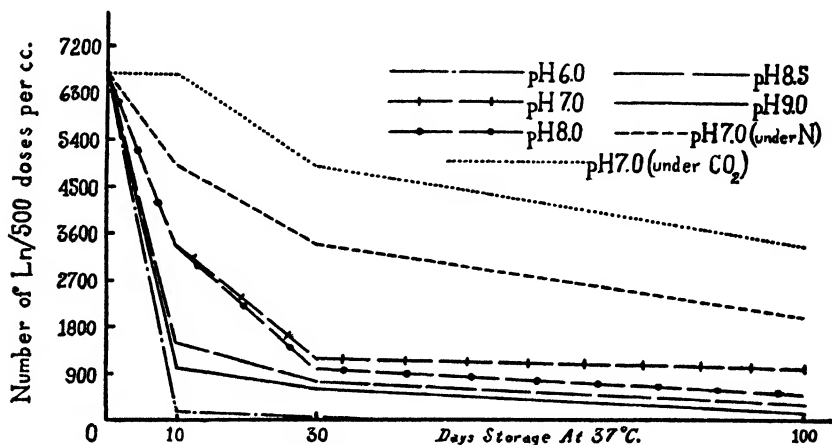sealed test tubes containing 10 cc. of air and 5 cc. of test material.

CHART 2.   SHOWING THE INFLUENCE OF H-ION AND CO_2 ON THE STABILITY
OF DIPHTHERIA TOXIN

Samples of A adjusted to pH 8.8 and pH 7.0 were also stored
under similar conditions.   A temperature of 37°C. was selected
for the storage of the test toxin solutions in order to accentuate
the detoxifying process and thereby shorten the time necessary
for the experiment.

The results of this experiment are recorded in table 4 and are
expressed graphically in chart 2.

[3] All test tubes (6 inches by ½ inch) used in the storage experiments were treated
with hot chromic acid cleaning solution and then thoroughly rinsed with tap water
and finally with distilled water.   After drying, the tubes were heated near the
center and drawn out in such a way as to result in a constructed area 2 to 3 mm.
in diameter.   Five cubic centimeters of the test filtrate were then placed in each
tube with the aid of a special pipette.   The remaining space inside the test tubes
was filled with air, nitrogen or carbon dioxide.   The tubes were then hermetically
sealed by heating the constricted portion in a Bunsen burner flame.

Filtrates stored under CO_2. Practically no destruction of toxin occurred in portions of filtrates A, B and C which were stored under carbon dioxide for a period of thirty days. Portion A suffered only a comparatively slight decrease in toxicity after one hundred eighty days storage at 37°C.

A somewhat greater decrease in potency occurred in the portions of filtrate B which were stored for one hundred eighty days at 37°C. than in the portions of the other two lots of filtrates which were stored under carbon dioxide. This may have been due to the presence of sufficient carbon dioxide to cause a somewhat unfavorable reaction (pH 6.8). The above condition emphasizes the fact that, while carbon dioxide in limited amounts will preserve diphtheria toxin, enough carbon dioxide to increase the hydrogen ion concentration below pH 7.0 is harmful.

Filtrates stored under nitrogen. Unadjusted portions of filtrate B (pH 8.5) which were stored under nitrogen gradually decreased in toxicity during the first thirty days of storage, and became entirely atoxic at the end of seventy days storage at 37°C. In this case destruction of the toxin was apparently due either to the direct action of OH ions, or to the effect of dissolved oxygen which was not removed from the test portions of filtrate during the process of replacing the air in the containers with nitrogen.

The toxicity of filtrate C which was adjusted to pH 7.0 and stored under nitrogen decreased comparatively slowly during one hundred days storage at 37°C. The stability of diphtheria toxin under this condition was second only to that stored under carbon dioxide.

Filtrates adjusted to various hydrogen ion concentrations and stored in hermetically sealed tubes. Filtrates adjusted to pH 9.0: The toxicity of portions of filtrates A and B which were adjusted to pH 9.0 and stored at 37°C. in hermetically sealed tubes decreased rapidly, the test material becoming entirely atoxic by the end of thirty days. For some unknown reason, filtrate C (pH 9.0) remained slightly toxic after one hundred days storage. A similar observation has been reported by Heineman and Hixson (1922), who state that not all toxins deteriorate at the same rate.

Filtrates adjusted to pH 8.5: Portions of filtrate B which were

adjusted to pH 8.5 became atoxic at the end of seventy days, while the toxicity of the corresponding portions of C was not completely destroyed by the end of one hundred days.

Filtrates adjusted to pH 8.0: Decrease in toxicity was approximately the same in the portions of filtrates B and C which were adjusted to pH 8.0 as in the portions of the same filtrates which were adjusted to pH 8.5.

Filtrates adjusted to pH 7.0: Portions of filtrate which were adjusted to pH 7.0 remained toxic for a longer period of time than portions of the same filtrates which were adjusted to any other hydrogen ion concentration. Even so, a marked destruction of toxin occurred in filtrates A, B and C, particularly in A, by the end of thirty days storage at 37°C. Filtrate B was completely detoxified by the end of seventy days, and filtrate A by the end of one hundred eighty days, while filtrate C remained fairly toxic after one hundred days storage.

Filtrates adjusted to pH 6.0: At pH 6.0, destruction of diphtheria toxin was very rapid. Filtrates B and C were but feebly toxic after ten days, and were completely detoxified by the end of seventy days storage at 37°C.

DISCUSSION

The data presented in the foregoing experiments show that carbon dioxide under suitable conditions may act as a preservative for diphtheria toxin. This preserving action is undoubtedly due primarily to two factors: namely, (1) the effect of carbon dioxide on the reaction of the toxic culture filtrate and (2) the prevention of the detoxifying action of molecular oxygen on diphtheria toxin. The first factor is supported by the observation that toxin solutions adjusted to pH 7.0 and stored in the presence of air or nitrogen remained potent for longer periods of time than other portions of filtrate which were adjusted to any other reaction and stored under similar conditions. The second factor appears to be substantiated by the fact that filtrates aerated with CO_2-free atmospheres containing 50 per cent oxygen decreased rapidly in toxicity; whereas duplicate portions of the same lot of filtrate having the same reaction did not decrease

in toxicity when aerated with an atmosphere containing 5 per cent carbon dioxide and 50 per cent oxygen.

The preserving effect of carbon dioxide has also been demonstrated for alexin by Valley and McAlpine (1928) and Valley (1928). These investigators state that the preservative action of carbon dioxide gas (under normal atmospheric pressure) is in all likelihood due to the establishing and maintaining of conditions which favor reduction and prevent oxidation.

CONCLUSIONS

1. Carbon dioxide in suitable amounts prevents the detoxifying action of molecular oxygen on diphtheria toxin.

2. Within the limits of pH 7.0 and pH 9.0, the lower the hydrogen ion concentration of the toxin solution the more rapid is the rate of destruction of diphtheria toxin by molecular oxygen (in the absence of carbon dioxide).

3. Diphtheria toxin may be preserved over comparatively long periods of time at a temperature of 37°C. when placed under carbon dioxide, providing sufficient CO_2 to produce an acid reaction is not present.

4. The toxicity of filtrates stored under nitrogen decreases less rapidly than does then toxicity of solutions having the same reaction but stored in the presence of a small quantity of air. Even so, the rate of decrease in potency is greater under nitrogen than under carbon dioxide.

5. The optimum hydrogen ion concentration for the preservation of diphtheria toxin is pH 7.0.

6. The preservative effect of carbon dioxide on diphtheria toxin in cultures grown under an increased carbon dioxide tension is accomplished by controlling the reaction of the culture medium and by preventing the oxidation of the toxin by molecular oxygen.

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A NOTE ON THE APPLICATION OF BUCHANAN'S FORMULA TO HEAT PRODUCTION IN BACTERIAL CULTURES

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This note is presented in order to discuss the results which Bayne-Jones and Rhees (1929) report in applying Buchanan's formula to their data on the production of heat in bacterial cultures. Unfortunately, the value of these observations is impaired by the fact that the mathematical treatment of the data is not entirely free from error. Apart from this, however, it appears desirable to reconsider the analytical aspects of the problem especially because the rate of heat production of a single bacterium cannot, contrary to the authors' claim, be represented in their experiments by the Buchanan formula.

They have based their claim entirely upon a striking numerical agreement between values calculated from the formula and those determined somewhat more directly from the individual measurements. The proof that this agreement is unreal will prepare the way for the demonstration that the liberation of heat had not taken place in accordance with the hypothesis from which Buchanan's formula has been derived.

We may begin our investigation of the matter by quoting from the original paper:

> The heat produced by each bacterium was obtained directly by dividing the total gram calories by the number of bacteria present in the 100 cc. of culture medium.

This clearly defines one of the units with which we shall be required to deal, namely, the quantity of heat liberated *per bacterium*. Thus calculated, it has been considered an "ob-

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served" value, since it was determined from the heat, and from the number of organisms experimentally measured and counted, respectively, at the end of each time interval,—in the present case, one hour. The highest value has consistently been found at the end of the second hour, and appears to be taken without further qualification as the true maximum of the function. Interpolation, however, on both growth and heat curves shows that the maximum is reached considerably earlier than the second hour. This needs to be mentioned only because the authors themselves have considered the discovery of points of inflection a rather significant result of their analysis. What concerns us more at the moment, however, is the fact that one of the units entering into the discussion has now been defined as *heat per bacterium*.

The sentence following that already quoted reads:

This value, of course, was actually too small to be measured but some interesting relationships were discovered through the graphic use of it.

The preceding statement thus refers unmistakably to the quantity heretofore defined as *heat per bacterium*.

We are, thereupon, informed that,

The same fact could be reached by a calculation.

The foregoing quotation obviously, can be interpreted only to mean that the dimensions of the quantity calculated from the formula which the authors are about to derive will be identical with the dimensions of the unit previously defined, that is to say, with *heat per bacterium*. This, however, is not the case, as we shall see by repeating the significant steps of the argument somewhat more briefly than in the original. It is assumed:

(*a*) That "the organisms are multiplying in geometric progression at a definite rate";

(*b*) That "each organism is excreting substance or (producing heat) at a definite uniform rate";

(*c*) That heat is measured at the end of each unit of time;

(*d*) That bacteria are counted at the beginning and at the end of each unit of time.—

Now, if, B = number of bacteria at onset,

b = number of bacteria at t,

g = average generation time,

S = total heat produced in t,

and, m = amount of substance or heat produced per cell per unit of time,

then, by condition (a) above,

$$b = B2^{\frac{t}{g}} \qquad (1)$$

if the "ratio in the geometrical progression" is 2.

Next follows the statement that "the amount of substance (or heat) produced during any instant dt would be $m\,b\,dt$," from which, (if this is true),

$$S = \int_0^t m\,b\,dt = mB \int_0^t 2^{\frac{t}{g}}\,dt \qquad (2)$$

In view of some of the results which the authors have announced, it is important in the first place to note that m has been removed from under the integral sign along with the constant B, this being justified, of course, on the basis that "each organism is excreting substance, (or producing heat) at a definite uniform rate." The foregoing procedure and definition thus afford absolute evidence that m is to be understood constant. Here is a conclusion of the utmost importance to which we shall need to refer at a later stage of our discussion. But there is another matter that should also be carefully noted at this time. While it is true that the above equation gives us a perfectly plausible relation between total heat, the number of organisms expressed as a function of the time, and time itself, we must bear clearly in mind that this relation is by no means a necessary one. Put into words equation (2) tells us that S, the total heat liberated in time t, has been supposed directly proportional to the number of organisms present in the culture medium. As we have already pointed out, however, the results of the present study do not support that hypothesis; but, rather than complete our examination of this matter here we may proceed instead to evaluate the

right hand side of (2), which becomes upon integration, substitution from equation (1), and rearrangement of terms,

$$m^* = \frac{S\ 2.303\ \log_{10} \dfrac{b}{B}}{t(b - B)} \tag{3}$$

This equation is known as Buchanan's formula, and serves as the basis for the "theoretical" determinations of the unit rate of heat production. Accordingly, when the authors now propose to demonstrate the applicability of this formula to their data by a comparison of the results so obtained with those determined by experiment, we should expect that the values of m would be compared with values representing the unit rate of heat production estimated from direct observation of heat output. Unfortunately, however, this has not been done. What has happened is that the values calculated from this formula have instead been compared with those values which we have already shown to be expressed in terms of *quantity of heat per bacterium* and not in terms of *rate of heat production per bacterium*. Here we refer specifically to the comparison drawn up in the last two columns of each of the original tables. Take for example[1] Table 1, which we have reproduced, in order to facilitate a ready check on what follows. We have designated the columns serially from left to right (1–4), and below each column we give its symbolical equivalent, where S, b, and t have the same significance as before. Column 3 as stated in the original heading, represents the *amount of heat produced per bacterium*, $\left(\dfrac{S}{b}\right)$, these values having been computed from the data given in columns 1 and 2. The values in column 4, however, which are compared in the text with those in column 3 have been obtained by calculation from equation (3) and thus represent the *rate of heat production per bacterium*.

* The original paper gives M (capital), apparently an error in proof-reading. By actual check m is the only quantity that can possibly be intended here. There is no indication for a change in symbols, especially one not already defined.

[1] Similar remarks apply to the experiments given in Tables 2, 3 and 4 of the original paper.

They do not represent, as the original column heading erroneously states, "Gram calories per Bacteria."[2] All of this is rather confusing inasmuch as we are led to believe from the original definition of m, that we shall meet with values representing *heat per bacterium per unit of time.* Having satisfied ourselves, how-

TABLE 1[*]

TIME	1 TOTAL HEAT	2 TOTAL NUMBER BACTERIA $\times 10^6$	3 GRAM CALORIES PER BACTERIUM OBSERVED $\times 10^{-9}$	4 "GRAM CALORIES PER BACTERIUM CALCULATED" $\times 10^{-9}$
hours	*gram Cal.*			
0	0.000	360	0.000	0 000†
1	1.485	420	3 540	4 608‡
2	17.680	2,160	8 182	8.770
3	50 290	9,600	5 240	5 950
4	76 650	33,600	2.280	2.650
5	92 730	96,000	0.966	1 080
6	108.600	129,000	0 842	0.829
Symbolic equivalent...	S	b	$\dfrac{S}{b}$	$\dfrac{S}{\displaystyle\int_0^t 2^{\frac{t}{g}}\,dt}$

* Recompiled from table 1 of the original paper (1).

† Strictly taken this value is indeterminate and not equal to 0. See equation (3).

‡ Our result = 3.77×10^{-9}. For explanation see footnote 3.

ever, by actual check that the values in column 4 have been calculated by means of equation (3) we see that this column accordingly gives,[3]

$$\frac{S}{B\displaystyle\int_0^t 2^{\frac{t}{g}}\,dt}.$$

[2] Accurately quoted.

[3] We have been unable to check the result given for the 1st hour, that is, 4.608. Our result is:

$$\frac{1.485 \times 2.303 \log_{10} \frac{420}{360}}{1 \times 60 \times 10^6} = 3.77 \times 10^{-9}$$

But, in spite of what has been said, it will be noted that there is a remarkable parallelism between the corresponding values in these two columns. This leads to still further confusion unless we keep clearly in mind what is actually being represented and compared. Granting, for the moment, that the foregoing correspondence is real, let us take the matter one step forward. Since the numerical agreement between these columns is of a high order,—differences being attributable to experimental errors,—we may equate the symbolical equivalents of these two columns, and we have,

$$\frac{S}{b} = \frac{S}{B \int_0^t 2^{\frac{t}{g}} \, dt}, \tag{4}$$

That is to say,

$$b = B \int_0^t 2^{\frac{t}{g}} \, dt.$$

But from equation (1)

$$b = B \, 2^{\frac{t}{g}}.$$

Hence,

$$B \, 2^{\frac{t}{g}} = B \int_0^t 2^{\frac{t}{g}} \, dt \; ! \tag{5}$$

Evaluating the integral as before, cancelling B on both sides,

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Compare, however, the result for the 2nd hour:

$$\frac{17.68 \times 2.303 \log_{10} \dfrac{2160}{360}}{2 \times 1800 \times 10^6} = 8.77 \times 10^{-9}$$

a value which checks with that given in the authors' table. Note that the value of B has to be taken as 360×10^6 in order to verify the authors' results. To do this neglects the fact that a slight but definite lag period preceded strictly logarithmic growth in this experiment.

collecting and rearranging terms, and finally dividing by $g2^{\frac{t}{g}}$ we get,

$$\frac{\log_e 2}{g} = 1 - 2^{-\frac{t}{g}}. \tag{6}$$

Now,

$$\frac{\log_e 2}{g} = \text{constant}.$$

But, the right hand side of (6) is not constant since it contains a function of t. This result proves beyond all question that the relation expressed in equations (4) and (5) is untrue, and we are, therefore, obliged to regard the comparison between columns 3 and 4 as illegitimate.

Our position now is about this: Critical examination of the text and tables has amply demonstrated that the comparisons just discussed are unsound, for it is perfectly clear that *heat production per bacterium* and *rate of heat production per bacterium* are two entirely different things, any numerical correspondence between them to the contrary. Furthermore, even if we should accept the tabulated agreement as significant, we could not escape the paradoxical conclusion that a variable is constant. This paradox is, of course, merely a necessary consequence of the fact already indicated, that the authors have in reality compared units of unlike dimensions.

At first glance it might appear possible to correct this error by dividing the values in column 3, which represent $\left(\dfrac{S}{b}\right)$, by t, thus obtaining a new set of "observed" values which would then be expressed in the same unit as that of column 4, namely, rate of heat production per bacterium. Actually, however, we should thereby not only destroy the numerical agreement heretofore existing between "experimental" and "theoretical" determinations, but we should also be making an obviously fruitless effort in trying to compare $\left(\dfrac{S}{bt}\right)$ "observed" with $\left(\dfrac{S}{\int_0^t b\,dt}\right)$ as given by equation (2).

The difficulty here depends chiefly upon the fact that the values obtained from $\left(\dfrac{S}{bt}\right)$, although expressed in the proper unit, constitute, as long as t is finite, only a very unsatisfactory approximation to the results given by $\left(\dfrac{S}{\int_0^t bdt}\right)$.[4] Thus, it is clear that the latter quantity cannot be taken as a measure of the accuracy in the former. Furthermore, if we are disposed to regard $\left(\dfrac{S}{bt}\right)$ as an "observed" value for the rate at which a single bacterium produces heat, we are likewise obliged to consider $\left(\dfrac{S}{\int_0^t bdt}\right)$ an "observed" rather than a "theoretical" or "calculated" value of m, since the elements entering into each of these expressions are as truly a matter of observation in the one case as in the other, the terms in the numerator even being identical. It is, therefore, apparent that we are not dealing in this particular instance with "observed" and "calculated" values at all, for this distinction applies only to results that have been independently obtained. Indeed, such a comparison is equivalent in many respects to an attempt at identifying a sharply-defined photograph by another of the same view which has been taken more or less out of focus. It would have been more acceptable, for instance, to have compared theoretical with observed values for S, since heat had actually been measured calorimetrically on the one hand, and could be calculated from the growth curves by equation (3) on the other, provided, of course, that m had been known rather than sought.

To sum up, we have now seen that the comparisons which the authors have made are clearly in error, and we have also seen that the comparisons which they apparently intended to make

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[4] Proof: Evaluate the integral in the denominator as before, expand the logarithmic term in the result on the condition that $\dfrac{b}{B} > 1/2$, and neglect quantities of an order higher than the first. This gives, $m = \dfrac{S}{bt}$.

do not serve their original purpose.   Accordingly, it can readily be understood why we have found it necessary to disagree with the statement that "the use of this equation is of great assistance in checking results and in reaching a decision as to the significance of unexpected values."   As a matter of fact, if S as well as b and B are subject to direct measurement, we prefer to look upon the formula merely as the method by which m can be precisely determined whenever heat is liberated during the logarithmic period in proportion to the number of organisms then inhabiting the culture medium.

We may now return to complete our inquiry into the significance of equations (2) and (3).   Broadly speaking, it is almost certainly true that the heat output in bacterial cultures is a function of their population, and we should, therefore, expect that variations in the liberation of heat would correspond more or less accurately to changes in the number of living organisms. The authors themselves have confirmed this general relationship, although the complete details have not been given since they have described the elimination of heat simply during the logarithmic phase of growth, in which it is not unreasonable to suppose the great majority of organisms living.   Under these conditions, a correlation between total heat and total number of bacteria is not open to serious question.   But the matter of distinct importance which here concerns us, is the precise nature of the mathematical relation between heat and bacterial growth. Bayne-Jones and Rhees, as we have seen, have assumed that each bacterium liberates heat at a constant rate and consequently they conclude that the total amount of heat is directly proportional to the number of organisms present in the culture medium. They have then employed the Buchanan formula, which has been derived on such assumptions, as an indirect method of calculating the unit rate of heat production, (which they cannot or at least have not observed) intending thereby to get additional information concerning the processes involved.   In this connection we have already called attention to the fact that, however reasonable the Buchanan formula may appear, there is nothing to prove that it represents a necessarily true relation between

heat production and bacterial growth. We have thus come to the point where we desire to bring forward the evidence upon which we base our own conclusion that the liberation of heat in the experiments under discussion has not been, and furthermore, cannot be, correctly expressed by equation (3).

In the first place, if observation and theory are on common ground, we must expect that the numerical values obtained from the right hand side of (3) will give a constant, or approximately constant value for m, since the rate of heat production per bacterium has previously been supposed "definitely uniform." An inspection of the tables and curves in the original paper, however, (we again refer to table 1 as an example), shows that the rate of heat production of a single bacterium has apparently been found to vary throughout the logarithmic period of growth,[5] rising from zero to a maximum *during* (not at the end of) the second hour, and thereupon declining gradually to the end of the experiment.[6] But it is perfectly clear that the foregoing results cannot be reconciled with the fact that the unit rate of heat production has not only been assumed constant, but, in addition, has actually been treated so. Indeed, we are in turn, inclined to regard this "variation" in the rate of heat production as *prima facie* evidence that bacteria do not liberate heat in accordance with the Buchanan formula, much as a change in the velocity constant of an apparently first order chemical reaction is interpreted to mean that the reaction fails to obey the monomolecular law. Nevertheless, in order to place this matter beyond any question whatsoever, it will be profitable, we believe, to consider the problem from another point of view.

To begin with, careful inspection of the original curves shows that heat output rises gradually for the first hour and then becomes almost exactly linear for a period of three to four hours

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[5] Even if this should prove to be true, it would not signify that "young bacterial cells produce more heat per cell than older ones."

[6] We are unable to agree that "a period of greatly reduced metabolic activity on the part of each bacterium begins at about the fourth or fifth hour and is maintained at a low constant rate for ten hours, and probably for a longer time," nor are we convinced that "the curves in the illustrations in this paper clearly show this prolonged constant heat production per cell."

or even longer. During this same period bacterial growth is practically logarithmic save for a slight but definite lag phase confined entirely to the first hour in the two experiments illustrated in figures 1 and 3 of the authors' paper. This linear relationship between heat output and time we especially desire to emphasize, because we are thereby obliged to conclude that the relation between these variables may reasonably be expressed, for the above interval in the form,

$$\frac{dS}{dt} = \text{constant.} \tag{7}$$

The simplicity of equation (7) merely implies that we are neglecting differences of the second and higher orders, since we are certainly not in a position, on the basis of the experimental data alone, to infer a more complicated, not to mention a more correct, expression for the rate of heat elimination. Viewed as a matter of observation, then, we may accept the rate of heat production as sensibly constant. But, when we turn, on the other hand, to examine the nature of the theoretical curve of heat production we find, in contradistinction to the foregoing result, that the rate at which heat has been assumed to be liberated is by no means constant, for differentiating (3) with respect to t, after substituting $B2^{\frac{t}{g}}$ for b and cancelling like terms, gives,

$$\frac{dS}{dt} = mB2^{\frac{t}{g}}, \tag{8}$$

an expression, as we may recall, from which the definite integral in equation (2) has been set up. According to (8), the rate of elimination must increase in geometric ratio with time, and it is, therefore, clear, that this equation cannot represent the phenomena to which the previous equation, (7) applies.

An effective demonstration of the foregoing results is given by the material here set up in table 2 and by the curves in figure 1, for which we have again used the data presented in the first table of the original paper. The observed values for S, taken from the third column of our table are nicely distributed about the

broken line in the lower portion of the figure. The theoretical values for S, on the other hand, lie upon the smooth curve that rises far too rapidly even within this relatively short 4 hour period. The latter values have been computed as follows:

1. With the aid of equation (1) in its logarithmic form,

$$\log_{10} b = \log_{10} B + \frac{t}{g} \log_{10} 2, \qquad (9)$$

we have calculated the mean values of B and g from the data

TABLE 2

TIME	NUMBER OF BAC-TERIA CALCULATED FROM EQUATION (9) $\times 10^6$	$m \times 10^{-9}$	HEAT OUTPUT (S)	
			Observed in experiment 1	Calculated from equation (3) when $m = 7.9 \times 10^{-9}$
hours			gram Cal.	gram Cal.
0	105 4	—*	0.000	0 00
1	457.0	6.15	1.485	1.90
2	1,990 0	13 75	17.680	10 15
3	8,640 0	8 64	50.290	46 00
4	37,400 0	3 02	76 650	200 50

* Indeterminate.

by the method of averages over the four hour period manifesting geometric growth, and have found,

$$\log_{10} B = 8.0228$$
$$B = 105,400,000 \text{ organisms}$$
$$g = 0.471 \text{ hour}$$

2. We have then computed theoretical values of b indirectly from (9) for substitution into equation (3); these lie upon the straight line shown in the upper portion of figure 1. It will be noted that the initially observed value of $\log_{10} B$ is situated at a considerable distance from this curve, a fact which shows that lag, though of short duration, would seriously interfere with the subsequent calculation of S.[7] We prefer, for the purpose of illustration, therefore, to use the value given above, since equa-

[7] Compare footnote 3.

tion (3) provides only for strictly logarithmic growth. The remaining values are, as we see, fully in accord with this provision.
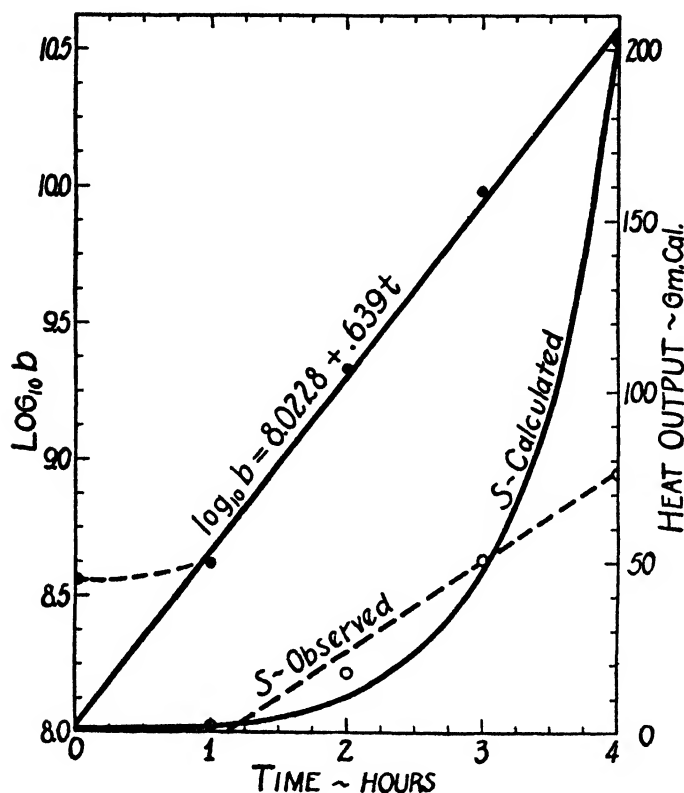

Fig. 1. The upper curve represents equation (9) fitted to the data for the first 4 hours, where $\dfrac{\log_{10} 2}{g} = 0.639$, and $\log_{10} B = 8.0228$. Note the presence of lag during the first hour.

The two lower curves clearly show the difference between total heat as measured experimentally and as expected according to the conditions defined by equation (3).

3. The foregoing values of b and B, with the corresponding values of t have then been substituted into equation (3) and the series of values for m determined, which are given in the second column of table 2. These, we note, do not agree numerically with similar values which the authors have obtained because of

the difference in the method of calculation to which we have just now referred. The average value for m is, 7.9×10^{-9} gm. calories per hour.

4. Upon resubstituting this value of m, as well as the above values of b, B and t into equation (3) we have finally calculated the theoretical values of S set up in column 4 of table 2 and lying, as we have already explained, upon the smooth curve in figure 1.

This curve rises somewhat too slowly at first, but during the last hour of a period that is very short indeed it shows such a tremendous increase that the final values at the end of the fourth hour differ from the observed heat output by approximately 200 per cent! The trend and the slope of the two curves clearly illustrate an increasingly conspicuous disagreement between theory and fact, in that the slope of the curve for the observed values is practically constant, whereas the slope of the theoretical curve can easily be seen to respond to a powerfully augmented function of time quite in accord with the provision of equation (8).

Thus, we are again brought to the conclusion that Buchanan's formula does not apply to the data under discussion. Moreover, since this equation fails to hold for S, it cannot be employed to calculate m. Consequently, if m is desired we need to proceed differently. Combining equation (1) with equation (7) we have,

$$m = \frac{1}{b}\frac{dS}{dt} = \frac{\alpha}{B}2^{-\frac{t}{g}} \tag{10}$$

in which α represents the value of the constant in (7). This is an important result since it proves that the curve of the unit rate of heat production is a simple diminishing exponential function of time as long as growth is logarithmic and heat output linear; it also shows that under these conditions, m does not possess either "critical" or maximal values. We must, therefore, distinguish clearly between m as given by equation (10) and the quantity $\frac{S}{b}$ which may be obtained from the relation

$$\frac{S}{b} = \frac{\alpha\,t}{B\,2^{\frac{t}{g}}} \tag{11}$$

in which the numerator of the right hand side represents the integral of (7) with the constant of integration set equal to 0, since the initial conditions provide that $S = 0$ when $t = 0$. Differentiating (11) once with respect to t and equating to 0, we have,

$$t = \frac{g}{\log_e 2}. \tag{12}$$

A second differentiation gives,

$$\frac{d^2\left(\frac{S}{b}\right)}{dt^2} = \frac{\alpha \log_e 2}{Bg} \cdot 2^{-\frac{t}{g}}\left(\frac{t \log_e 2}{g} - 2\right). \tag{13}$$

Upon substituting the value of t from (12) into (13) we have

$$\frac{d^2\left(\frac{S}{b}\right)}{dt^2} = -\frac{\alpha \log_e 2}{Bg} 2^{-\frac{1}{\log_e 2}} = -\frac{\alpha \log_e 2}{Bg \ \epsilon}. \tag{14}$$

where ϵ is the Napierian base of logarithms.

Hence, since $\dfrac{d\left(\frac{S}{b}\right)}{dt} = 0$ and $\dfrac{d^2\left(\frac{S}{b}\right)}{dt^2} < 0$ when $t = \dfrac{g}{\log_e 2}$, we may conclude that the curve for $\left(\dfrac{S}{b}\right)$ in contrast to the curve for m has a maximum, which is reached in the present example, .681 hours after the liberation of heat has begun.[8] Again, setting (13) equal to 0 we find,

$$t = \frac{2 g}{\log_e 2}. \tag{15}$$

Therefore, the point of inflexion of (11) is situated twice as far away from the onset of heat production as the maximum. It

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[8] In the example already discussed heat did not begin to be liberated until the end of the first hour. The maximum, therefore, is situated at about 1.68 hours or slightly before, and the point of inflexion at 2.36 hours.

will also be noted from (12) and (15) that the position of the maximum as well as that of the point of inflexion are independent of the rate at which heat is liberated, and vary only with the generation time of the culture. The more rapidly the organisms grow, the sooner the maximum and the point of inflexion are reached.

Thus, in reviewing our evidence as a whole, we may finally conclude that the Buchanan formula cannot be employed to calculate the unit rate of heat production in the experiments which Bayne-Jones and Rhees have performed. Support for this conclusion rests chiefly upon the demonstration that heat has not been liberated in accordance with the assumptions to which this formula may be traced. Incidentally, it has become evident that the unit rate of heat production is actually a diminishing exponential function of time whenever heat output during the logarithmic phase of growth is linear, two conditions, we may recall, that have been quite satisfactorily reproduced in the experiments to which we refer. This quantity neither remains constant, as the Buchanan formula demands, nor does it vary in the manner shown to be characteristic of the curve describing unit heat production. What bearing these facts may have upon our ultimate concepts in regard to the processes concerned with bacterial metabolism, we prefer to leave to further experimental investigation.

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THE FERMENTATION OF GLYCURONIC ACID BY CERTAIN BACTERIA

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Although glycuronic acid is structurally very closely related to glucose, and is, furthermore, apparently widely distributed in both the animal and plant world, it has received little attention in the biological sciences outside of physiological chemistry. Glycuronic acid is a true sugar acid, for it possesses a carboxyl group and still preserves the essential carbonyl or aldehyde group of a sugar. Structurally, it differs from glucose only in that the terminal alcohol group has been oxidized to a COOH group as shown by the following formulae:

Glucose   Glycuronic acid
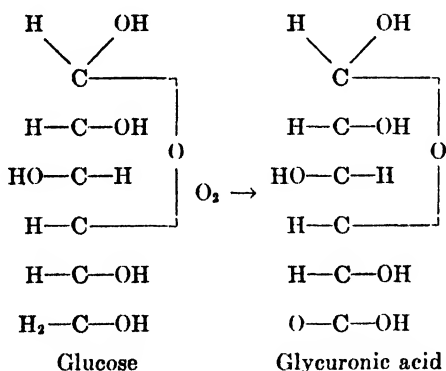
The close chemical relationship that glycuronic acid has to glucose immediately raises the question as to the position and importance of this compound in the intermediary metabolism of glucose. The general consensus of opinion seems to be that glycuronic acid does not represent a step in the normal metabolism of glucose. In the first place, the free or uncombined glycuronic

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acid does not appear to be handled readily by the animal organism, even though all laboratory animals, as well as man, can synthesize glycuronic acid in a conjugated form with ease and in relatively large quantities. In the second place, it appears that the introduction of the acid group into the glucose molecule has affected a profound change in its physiological behavior as illustrated by the fact that glycuronic acid is not fermented by yeast nor can it alleviate and cure insulin convulsions, according to Hurthle (1927).

In view of these facts it seemed interesting to investigate the action of bacteria on glycuronic acid. Such an investigation might not only furnish new information on the physiology of bacteria, but might also be valuable as an aid to evaluate metabolic studies in animals in which glycuronic acid was administered *per os*. Recently, Kay (1926) found that *B. coli* can utilize glycuronic acid and produce qualitatively the same end products as it does from glucose, but the relative amounts formed are markedly different. The author further states that glycuronic acid is fermented by a large number of bacteria of the colon-typhoid group but presents no experimental data nor specifies which particular organisms ferment it. It seemed desirable therefore to determine which particular bacteria can utilize glycuronic acid. Since glycuronic acid can readily be prepared in large quantities by a method recently developed by one of us (1927) it seemed worth while to investigate whether this compound might find a place in the list of sugars used in the differentiation of bacteria.

Glycuronic acid offers another point of interest to the bacteriologist. Recently, Heidelberger and Goebel (1927) found that glycuronic acid is one of the constituents in the serologically type-specific polysaccharide of the pneumococcus. The aldobionic acid which they obtained from the hydrolysis of the Type III specific carbohydrate was found to be a compound of glycuronic acid and glucose united in glucosidic linkage, and an isomeric compound of glycuronic acid and glucose was also obtained from the Type A Friedländer bacillus (1927). It thus appears that bacteria as well as the higher forms of life are capable of synthesiz-

TABLE 1

Fermentation of glycuronic acid by certain aerobic bacteria

ORGANISM	H-ION CONCENTRATION AFTER 48 HOURS INCUBATION
B. typhosus, No. 211 .	6 0
B. typhosus, Laboratory strain A	6.0
B. paratyphosus A, No. 235 M	6 2
B. paratyphosus B, No. 236 E	6 4
B. paratyphosus B, Laboratory strain S	6.4
B. coli-communis	6.1
B. coli-mutabili	6.3
B. coli-communior, Laboratory strain G	6 0
B. coli-communior, Laboratory strain 1 (a)	6 0
B. coli-communior, Laboratory strain 1 (b)	6 0
B. coli-communis	6.2
B. aerogenes, Laboratory strain No. 1	5 6
B. aerogenes, Laboratory strain No. 2	5 8
B. aerogenes, Laboratory strain No. 3 ..	5 8
B. aerogenes, Laboratory strain No. 4	5 6
B. dysenteriae (Flexner)	6.0
B. enteritidis, No. 273 .	6 4
Vib. cholerae, No. 581	6 0
B. alkaligenes .	7 1
B. proteus (Felix Weil)	6 1
B. proteus vulgaris, No. 23 ATE 2	6 5
M. zymogenes, Laboratory strain M	6 3
M. zymogenes, Laboratory strain S	6 2
B. fluorescens .	7.2
B. pyocyaneus, Laboratory strain H	7.2
B. pyocyaneus, Laboratory strain No. 1	7 2
B. prodigiosus, No. 274.	6 4
Staphylococcus aureus, No. 72	7.2
Staphylococcus aureus, No. 77	7.2
Staphylococcus aureus, Laboratory strain R	7.1
Staphylococcus aureus, Laboratory strain T .	7.1
Staphylococcus albus .	6.2
Streptococcus hemolyticus (Beta), from stool ..	7.1
Streptococcus viridans, No. P 7 H	6.5
B. subtilis .	6 0
B. anthracis, No. 10 ..	7.1
Oidium albicans	7 2
Control, sterile medium..	7 2

ing glycuronic acid. The desirability of knowing more about the metabolism of this compound by the higher forms of life as well as by bacteria is apparent.

A series of aerobic and of anaerobic bacteria were planted in sugar free broth containing 1 per cent of glycuronic acid. This medium was adjusted to pH 7.2. In the case of the aerobic organisms the plants were made from twenty-four-hour growths in 0.5

TABLE 2

Fermentation of glycuronic acid by certain anaerobic bacteria

ORGANISM	H-ION CONCENTRA-TION AFTER 48 HOURS INCUBATION
B. welchii (Bull and Pritchett)	6 0
B. welchii, Type 1	6 0
B. welchii, Type 2	6.1
B. welchii, Type 3	5.8
B. welchii, Type 4	6.5 ·
B. oedematiens	6.3
Vibrion septique	6.3
B. tetani (Pasteur)	6.5
B. aerofoetidis	6 6
B. sphenoides	6 0
B. putrificus (Meyer)	6.4
B. bifermentans	6 3
B. botulinus, Type A (U. S. P. H. S.)	6.0
B. botulinus, Type A (Burke)	6 3
B. sordellii	6.2
B. sporogenes	6.5
B. centro-sporogenes (Hall)	6.2
B. histolyticus	6 5
Control, sterile medium	7.2

per cent hormone agar. The anaerobic test cultures were planted from forty-eight-hour growths in casein digest broth and oxygen was excluded by use of the vaseline-seal boiling technic. Both anaerobic and aerobic test cultures were allowed to incubate at 37.5°C. for forty-eight hours, at the end of which time colorimetric tests were made to ascertain the hydrogen ion concentration for each individual culture.

SUMMARY AND CONCLUSIONS

The results obtained in the case of the aerobic organisms are summarized in table 1, and those for the anaerobes in table 2. In the case of the former it may be seen from this limited test that the members of the colon-typhoid-dysentery group are able to utilize glycuronic acid as a source of energy.   Four strains of *Staphylococcus aureus* did not, in the presence of this substance, reduce the H-ion concentration to any appreciable degree, while a single strain of *Staphylococcus albus* produced acid, as evidenced by reduction of the H-ion concentration from pH 7.2 to pH 6.2. Before this substance can be used for separative purposes, however, a greater number of representatives of various bacterial species will have to be tested and it is hoped that other workers will be led to make further determinations with this substance in the possible differentiation of some of the above-mentioned and other bacterial types.   Most of the anaerobic spore bearers which ferment glucose are also able to ferment glycuronic acid but seemingly less energetically so.   In this group, also, a few members which do not ferment glucose, as evidenced by a reduction of the hydrogen ion concentration, feebly utilize glycuronic acid.   These are *B. tetani* (Pasteur strain), *B. putrificus* (Meyers strain) and *B. histolyticus* (Barber-Weinberg strain).

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THE SPECIFICITY OF SCARLATINAL HEMOLYTIC STREPTOCOCCI

WITH SPECIAL REFERENCE TO THE FORMATION OF RASH DEVELOPING SUBSTANCES

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INTRODUCTION

Since the publication of a new conception regarding the etiology of scarlatina by Dick and Dick, strong support has been given to the view that this disease is streptococcal in origin; and numerous researches with regard to the relationship of hemolytic streptococci to the disease have been carried out in the four corners of the globe. The occurrence of hemolytic streptococci in the throats of patients with scarlatina was generally known but it was believed that streptococci might be absent in a certain form of the disease, especially in its foudroyant form. The most recent observations, however, indicate that hemolytic streptococci are almost constantly associated with this disease regardless of its severity. I myself studied 273 cases, including 42 cases of the foudroyant form, in Manchuria, China, employing direct pouring cultures in blood agar plate with the throat swabbings taken within five days after the onset of the disease. All cases resulted positively, with the exception of 7 cases which were surgical scarlatina. In 6 out of these 7 cases of surgical scarlatina, hemolytic streptococci were found in the suppurative lesion instead of in the throats. One case of surgical scarlatina nowhere harboured hemolytic streptococci but showed *Staphylococcus aureus* in the suppurative lesion. Stevens (1927) has described his own experience with such a case of scarlatini-form rash. Such a condition, however, is rare. Hemolytic streptococci therefore are found in

primary association with scarlatina even early in the acute stage of the disease.

Until 1923, experimental scarlatina had not been produced in animals or in persons by inoculation of a pure culture of any organism; but in this year, Dick and Dick succeeded in producing 2 cases of scarlatina in human volunteers by inoculation with an apparently pure culture of a hemolytic streptococcus which was isolated from a lesion on the finger of a nurse who had contracted scarlatina while caring for a convalescent scarlatina patient. Since then this finding has been repeated and confirmed by Nicolle, Consel and Durand (1926), and by Moriwaki (1928); all obtained experimental scarlatina by inoculation of scarlatinal hemolytic streptococci in the throats of human volunteers.

At the present time there is not the slightest doubt that anti-toxic-serum against scarlatinal hemolytic streptococci exerts remarkable influence on scarlatina. Therefore it is unnecessary to review the literature on the subject.

Active immunization with material from scarlatinal hemolytic streptococci is likely to protect persons from infection, as the results in Russia (Korschun, 1928), where Gabritschewsky's method was executed on a large scale, and my own recent statistical record of the result of active Dick immunization in Dairen have shown (Moriwaki, 1929).

Such being the case, the streptococcal etiology of scarlatina appears to fulfill Koch's postulates for recognizing a causative agent. Hemolytic streptococci, however, constitute a group of microörganisms etiologically related to various pathological conditions, and also occur as common commensals in the throats of healthy persons. No clear-cut characteristics that seem to differentiate scarlatinal streptococci from streptococci of other origins have been demonstrated, and because of this fact, hemolytic streptococci have long been regarded as nothing more than a secondary invader.

In the light of our present knowledge, which shows that scarlatinal symptoms can be produced by scarlatinal streptococci or the filtrate of their broth cultures, it is inadmissible to consider streptococci as merely secondary invaders. That the causative

principle of scarlatina may exist closely related with scarlatinal streptococci and that it may pass through bacterial filters is a well recognized fact.   It will require further studies, to prove whether there may not be an unknown virus which renders scarlatinal streptococci active as supposed by Zlatogoroff (1928), or a transmissible virus as supposed bv Brown and Frobisher (1927).

The microörganisms proposed by such authors as Caronia (1925), Smirnowa-Zamkowa (1926), and Mandelbaum (1927) seem to have no demonstrated existence from a primarily etiological point of view, unless it is proved that they also produce scarlatinal symptoms by themselves, since the filtrate of pure broth culture of scarlatinal streptococci actually contains a scarlatinal antigenic principle.   This principle seems to be considered as an extracellular toxin of the streptococci by Dick and Dick, and as an allergen produced by streptococci according to Dochez. I will use hereafter in this article the term "toxin" for this principle for convenience sake.

I made an experimental study with regard to the specificity of scarlatinal hemolytic streptococci, with special reference to the rash-developing possiblity.   Such an approach, which has never been tried, promises to throw further light on the etiology of scarlatina.

STRAINS INVESTIGATED

All the scarlatinal strains employed in the present research were hemolytic streptococci of β type, strictly speaking the βt type of Ando (1922–23; 1923–25).   The strains retained Gram positiveness through generations contrary to the findings of Renc (1927) and of Bliss (1922).   The microörganisms did not pass through Chamberland F, B, L_3, L_5 or Berkefeld V filters.   For comparison, hemolytic streptococci of βt type isolated from pathological lesions other than scarlatina, and from healthy throats were also used.

CARBOHYDRATE FERMENTATION

The medium used in determining the fermentation powers was Hiss serum water of pH 7.0 to 7.2 containing 1 per cent of the

carbohydrates with bromthymolblue as indicator. After inoculation, the media were incubated for seven days at 37°C. Among 97 scarlatinal strains, (75 strains isolated from throats, 12 from adenitis, 5 from otitis media, 4 from heart blood, 1 from a surgical case) 66 were *Str. pyogenes*, 17, *Str. infrequens*, 8, *Str. anginosus*, 3, *Str. equi*, 2, *Str. haemolyticus* I, and 1 *Str. haemolyticus* II, according to Holman's method of classification. Seventeen strains of non-scarlatinal origin (4 strains from phlegmon, 3 from suppurative lesions, 3 from erysipelas, 2 from paronychia, 2 from rhinitis, 1 each from gangrene, myositis and adenitis) all belonged to *Str. pyogenes*. Fifteen strains from the healthy throats also fell to *Str. pyogenes* but 5 strains among them fermented raffinose, which classes them in the group of *Str. salivarius*.

<div align="center">AGGLUTINATION</div>

The serological relationship of scarlatinal streptococci to other hemolytic streptococci has been extensively studied lately by Stevens and Dochez (1926), Tunnicliff (1926), Mackie and McLachlan, Smith (1926), and Griffith (1926). Dick and Dick (1924) mentioned that there were at least 2 serological varieties showing no cross agglutination. Varieties have also been observed by others. Stevens and Dochez indicated that scarlatinal strains formed a fairly well defined group. Dick and Dick, however, are of opinion that the agglutination test is not to be relied on for the identification of scarlatinal strains. It is therefore still obscure whether scarlatinal streptococci separated etiologically fall into a group or groups entirely distinct from other hemolytic streptococci. I used serum from scarlatinal patients. The microörganisms were cultivated in broth of pH 7.4 without sugar for eighteen hours, (strains showing spontaneous clumping in the medium were discarded) the medium was removed by centrifugalization and the organisms were washed twice in distilled water, and resuspended in normal saline of double the original volume of the medium, and were then ready for use in the test. Sera of 8 patients, in early as well as convalescent stages, were tested with strains from scarlatina, erysipelas, puerperal fever, and healthy throats. Convalescent serum agglutinated scarlati-

nal strains in the dilution 1 in 40 to 1280 whereas the serum from the same person in the early stage of the disease hardly agglutinated in the dilution 1 in 40.   Homologous strains were agglutinated markedly in high dilution.   The strains from puerperal fever, erysipelas, and healthy throats were also agglutinated by the convalescent sera, frequently in as high dilution as the scarlatinal strains.

PRECIPITATION

The precipitation tests consisted of layering cleared filtrates of twenty-four-hour-old cultures of hemolytic streptococci in 0.5 per cent glucose broth over sera of scarlatina patients in Uhlenhuth's, tubes, and noting the presence or absence of a cloud at the juncture of the filtrate and the serum after twenty minutes' incubation.   Twenty out of 45 scarlatinal strains showed positive reaction with convalescent sera whereas 30 sera of early stage were all negative.   The convalescent sera also showed positive reaction in 18 out of 90 instances with non-scarlatinal strains (Erysipelas, 7 positive in 30 instances; panaritium, 2 in 15; gangrene, 3 in 15; strains from healthy throats, 6 in 30.)

COMPLEMENT FIXATION

The antigen was prepared in the same manner as that used in the precipitation test.   The convalescent sera fixed complement in 63 instances out of 65 with scarlatinal strains while that of early stages showed no fixation.   The convalescent sera, however, showed fixation, in 31 instances out of 44 with erysipelas strains, in 21 out of 63 non-scarlatinal strains isolated from phlegmon, panaritium, myositis and rhinitis, and in 1 instance out of 16 with strains from healthy throats.

TOXIN FORMATION

Dick and Dick demonstrated the production by scarlatinal hemolytic streptococci of an extracellular toxic principle capable of developing a cutaneous reaction in non-immune persons. I made an experiment in order to ascertain the toxigenic power of a large number of scarlatinal streptococci strains and at the same

time to make clear whether hemolytic streptococci from other sources than scarlatina have such toxigenic power or not. One hundred and fourteen strains associated with scarlatina, and 17 from various other sources were employed. The strains were cultivated in 0.5 per cent glucose broth of pH 7.2 for four days, and the toxin was obtained by filtration through Chamberland F, L₃ or Berkefeld V. The filtrate, diluted to 1 in 1,000 and 0.1 cc., was intradermally injected into the forearm. Each toxin was tested on over 10 children. The result was read after twenty-four hours. Those strains which gave almost similar or stronger reactions as compared with that of 1 s.t.d. of the standard toxin

TABLE 1

Toxigenic power of scarlatinal strains

STRAINS ISOLATED FROM	STRAINS TESTED	TOXIN PRODUCERS
Throats.........	70	65
Complicated lesions:		
Adenitis.........	18	16
Otitis media...	11	9
Mastoiditis	4	3
Heart blood.........	4	4
Throats of healthy persons in contact with patients..	5	4
Dust of patients' room	4	2
Total........	116	103

were designated as toxin producers. The standard toxin consisted of Chamberland F filtrates mixed in equal proportion with four days culture of 5 scarlatinal strains, in 0.5 per cent glucose broth; 2 strains were kindly sent by G. F. Dick and the other 3 strains were isolated in Dairen.

An intradermal injection of 0.1 cc. of a 1 in 1,000 dilution of the mixture gave almost parallel result with 1 s.t.d. of the standard solutions of Parke Davis and Company, and of Wellcome Physiological Research Laboratory (which was kindly sent by Dr. Okell). The positiveness of my standard 1 s.t.d. was 41.3 per cent among 13,029 healthy persons aged from one to fifty, 16.7 per cent among 419 healthy persons who had a previous history of scarlatina, and 4 per cent among 316 convalescent scarlatina patients.

A large number of scarlatinal strains tested were found to be toxin producers as shown in table 1.

However, the strains from other sources such as erysipelas, puerperal fever, phlegmon, gangrene, paronychia and myositis were also found to be toxin producers as shown in table 2.

NEUTRALIZATION

The technique of the neutralization experiments was as follows: hemolytic streptococci were grown in 0.5 per cent glucose broth for four days and the culture was passed through Chamberland F or L_3 filters.   The filtrate was diluted to 1 in 100, and 0.1 cc. of the diluted filtrate was mixed with an equal part of the serum of a

TABLE 2

Toxigenic power of non-scarlatinal strains

STRAINS ISOLATED FROM	STRAINS TESTED	TOXIN PRODUCERS
Erysipelas..	8	8
Puerperal fever......................................	2	2
Phlegmon.............	3	2
Gangrene...	1	1
Paronychia.........	2	2
Myositis	1	1
Total.........	17	16

scarlatinal patient and 0.3 cc. of normal saline; the final dilution of the filtrate was therefore 1 in 500.   The mixture was incubated for an hour and then 0.1 cc. was intradermally injected as usual. Each toxin was tested on over 4 children.   The filtrate alone in a dilution of 1 in 500 was employed as a control.   The result was read after twenty-four hours by comparing with the control and noted as following: Completely immunized $(+++)$ when the reaction of the mixture was entirely negative while that of the control was positive; not neutralized $(-)$ when the two reactions were of the same positiveness; incompletely neutralized $(++)$ and $(+)$ when ranked between $(+++)$ and $(-)$, according to the relative ratio of the two reactions.

The result was as follows: Strains from scarlatinal source were neutralized by convalescent serum, in 41 out of 59 tested (67.9 per cent), the actual figures being $(+++)$ 7, $(++)$ 16, $(+)$ 18 and $(-)$ 18.

Strains from erysipelas were neutralized in 12 out of 17 instances tested (66.6 per cent), the results being $(++)$ 7, $(+)$ 5 and $(-)$ 6.

Strains from phelgmon and gangrene were neutralized in 5 out of 16 instances (31.2 per cent), $(++)$ 4, $(+)$ 1 and $(-)$ 11. Strains from healthy throats were neutralized in 9 out of 28 cases tested (32.1 per cent), $(++)$ 3, $(++)$ 2, $(+)$ 4 and $(-)$ 19.

No neutralization was observed with sera of patients in an early stage of the disease. The formation of the toxin which is neutralizable by convalescent sera is not, therefore, limited to scarlatinal hemolytic streptococci but common to hemolytic streptococci from other sources, to a certain extent. The same result was arrived at also by other investigators such as Williams (1925) (with convalescent sera), Kirkbride and Wheeler (1926), Eagles (1924), McLachlan (1927), Lash and Kaplan (1926), Pilot and Westlund (1927), Birkhaug (1925), and Ando and Kurauchi (1928) (with antitoxic sera).

Even though the formation of the toxin is not specific to the scarlatinal hemolytic streptococci, Dick's skin test, however, might empirically be an index of susceptibility to scarlatina as statistically viewed from a practical standpoint. What is then the nature of the Dick's skin test,—a toxic reaction such as Schick's reaction in diphtheria or an allergic reaction such as that to tuberculin? Dochez and Stevens (1927), Kirchner (1928), Zinsser and Grinnel (1925), Mackie and McLachlan (1927), and Moriwaki (1928) obtained positive skin reactions in experimental animals after previous treatment with streptococci.

Cooke (1926) reported that new-born infants show a negative skin test irrespective of the presence or absence of antitoxin in the blood, and further that the offspring of a mother of positive skin reaction may show a negative skin reaction. On testing Schultz-Charlton's phenomenon in sera of 31 mothers and their new-born infants, I have proved that antitoxin content in the sera of a mother and her offspring are almost of the same grade.

Since new-born infants are generally of negative skin reaction it must be admitted that the skin reaction is not an index for presence of antitoxin in new-born infants. However, skin reaction and antitoxin content appear to be in a definite correlation in older individuals, as shown by the author, and by Joe (1925), although it is uncertain whether they necessarily bear a direct relation to each other as in diphtheria.

New-born infants exceptionally develop typical scarlet fever, as do more aged individuals of negative skin test. Therefore, negative reactors can be said to be free from the possibility of an attack of typical scarlatina.

SCHULTZ-CHARLTON'S BLANCHING PHENOMENON

Schultz-Charlton's rash blanching phenomenon is specific for scarlatina. That sera of experimental animals properly immunized with scarlatinal hemolytic streptococci produce blanching is an important fact made clear after the appearance of the new conception of the streptococci etiology. Hemolytic streptococci from other sources however, are also capable of giving a blanching property to the sera of animals on proper immunization. Thus, the fact that antitoxic sera of erysipelas hemolytic streptococci exert a blanching effect is generally known. Futagi (1927), a co-worker of mine, succeeded in obtaining blanching serum in rabbit by immunizing after Dochez's procedure with hemolytic streptococci isolated from gangrene. Boente (1927), Zlatogoroff (1928), Friedemann and Deicher (1928) also described animal sera capable of developing blanching, obtained by immunizing with streptococci from other origins than scarlatina. Antigenic property as shown in the Schultz-Charlton's phenomenon, is not, therefore, specific to scarlatinal strains, but common to hemolytic streptococci as a whole, to a certain extent.

The facts suggest that antitoxic sera prepared with strains not associated with scarlatina will be also efficacious in scarlatina. Boente's clinical observations endorse this suggestion. I myself used erysipelas antitoxic serum in scarlatina and obtained the same influence on rash and other initial symptoms of scarlatina, as shown in figure 1 which is a representative case.

LOCAL IMMUNITY TO SCARLATINAL RASH BY TOXIN OF HEMOLYTIC STREPTOCOCCI

Filtrate of a broth culture of scarlatinal hemolytic streptococci injected intradermally in a person, may show a peculiar phenomenon at the site of the injection when the person later develops
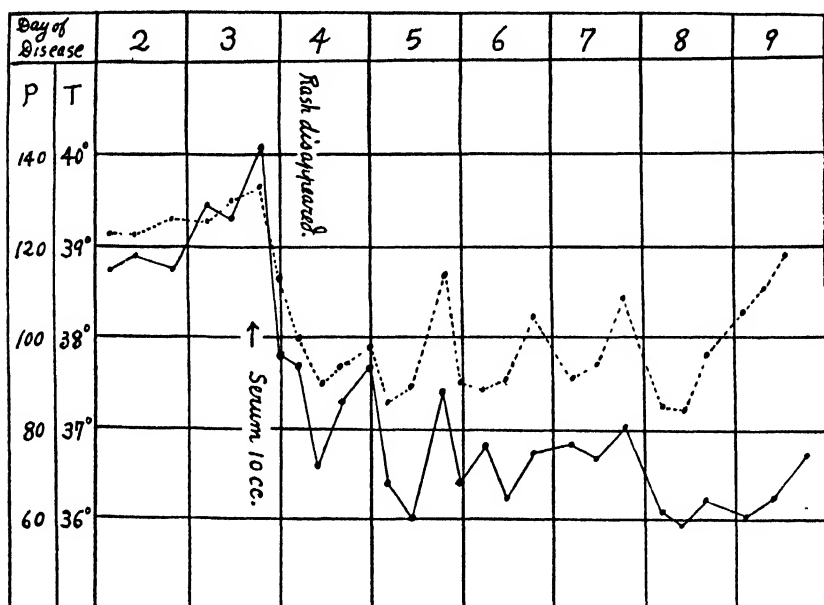

FIG. 1. TEMPERATURE IN A SEVERE CASE OF SCARLET FEVER TREATED WITH ERYSIPELAS ANTITOXIN

A boy four years old; 10 cc., of antitoxic horse serum against hemolytic streptococci of erysipelas origin was intramuscularly injected, on the third day of the disease when the rash was most intensive on the whole body; the rash completely disappeared on the following day, temperature also became normal, and the patient got well without any complication. Desquamation was weak.

scarlatina. Namely, about the site of the injection the rash may be entirely absent or may be more marked than in the other parts of the body. Sometimes the area about the site of the injection may be free from rash in the center and markedly reddened at the periphery. These phenomena have been already described by Toomey (1926), Ferry (1926), Zingher (1924), and Moriwaki

(1927), and the explanation offered of local immunity or allergy. I observed, however, the same phenomenon in persons previously intradermally injected with toxin of streptococci from puerperal fever and erysipelas when they contracted scarlatina. The area was more brightly reddened in the 2 cases and free from rash in another case as shown in table 3.

TABLE 3

Local change of area treated with toxin of non-scarlatinal streptococci in presence of scarlatinal rash

NAME	AGE	PREVIOUS TREATMENT					TIME INTERVAL BETWEEN TREATMENT AND OCCURRENCE OF RASH	CHANGES (DIAMETER)
		Date	Site	Toxin (dilution)	Dose	Reaction (diameter)		
M. I.	2	December 24	Left forearm	Puerperal (1:1,000)	0.1	0.7 cm. reddened	42	1.5 cm. reddened
C. I.	7	December 24	Left forearm	Erysipelas (1:1,000)	0.1	0.5 cm. reddened	43	1.0 cm. reddened
S. O.	16	December 16	Left forearm	Puerperal (1:1,000)	0.1	2.5 cm. reddened	34	4 cm. blanched

In the first two cases, the previously treated area became inflamed as a forerunner of the scarlatinal rash.

In the third case, the rash was entirely blanched at the area previously treated.

In this respect, toxin of scarlatinal hemolytic streptococci does not show any difference from toxin of hemolytic streptococci of other origin.

INFLUENCE OF INJECTION OF TOXIN OF NON-SCARLATINAL HEMO-LYTIC STREPTOCOCCI ON SKIN REACTION

Injection of toxin of scarlatinal hemolytic streptococci can turn a positive Dick's skin reaction to a negative one or to a less positive one. As shown in table 4, I injected toxin of non-scarlatinal hemolytic streptococci in children whose Dick's skin reaction and also skin reaction to the toxin to be injected were known, and

found by retest after several injections that positiveness to Dick's skin reaction, as well as to the skin reaction to the injected toxin, was lessened at the same time.

The observation shows that the antigenic property which leads a positive skin reaction to become negative is common among non-scarlatinal hemolytic streptococci to a certain extent.

TABLE 4

Influence of injection of non-scarlatinal streptococci toxin on skin reaction with scarlatinal streptococci toxin

NAME	AGE	SKIN REACTION WITH		TOXIN INJECTED		RETEST WITH	
		Standard 1 s.t d.	Toxin used in injection (1:1,000)	Source	Amount	Standard 1 s.t.d.	Toxin used in injection (1:1,000)
Hase.	6	++	++	Gangrene	3.7	±	—
Aik.	2	++	+	Gangrene	2.0	±	—
Aic.	3	++	++	Gangrene	2.0	±	±
Air.	3	+	++	Erysipelas	3.5	—	—
Hig.	8	+	+	Erysipelas	3.5	—	—
Hir.	8	+	+	Puerperal fever	3.5	±	±
Hat.	5	+	+	Puerperal fever	2.7	±	—
Sum.	6	++	+	Healthy throat	2.5	+	±

Injection was intramuscularly done. Toxin was used in original concentration.

Retest was done seven days after the fourth injection. Result of skin test was described as follows:

(−) no reaction.

(±) reddened area of diameter of smaller than 0.5 cm.

(+) reddened area of diameter of about 1.0 cm.

(++) reddened area of diameter of about 2.0 cm. accompanying swelling.

SKIN REACTION WITH TOXIN OF NON-SCARLATINAL HEMOLYTIC STREPTOCOCCI ON RABBITS PREVIOUSLY TREATED WITH TOXIN OF SCARLATINAL STRAIN

Among a series of rabbits treated with the toxin of scarlatinal hemolytic streptococci, the skin reaction became positive sixteen days after the subcutaneous injection of 10 cc. of the toxin; in this rabbit, it was noted, as shown in the table 5, that a skin reaction with non-scarlatinal hemolytic streptococci, such as erysipelas and puerperal fever, also became positive at the same time.

Thus, efforts to identify scarlatinal streptococci by general biological characters have been unsuccessful, and in these respects scarlatinal strains show no essential difference from similar organisms isolated from various sources. Dold (1927) also failed to show distinct differences by his "Gewebsbiologisches Verhalten." Dick (1927) produced a sore throat by inoculation of streptococci from erysipelas in a person which resembled that caused in the same way by scarlatinal streptococci. By the experimental

TABLE 5

Parallel appearance of skin reactions caused by toxin of scarlatinal and non-scarlatinal hemolytic streptococci in a rabbit previously treated with scarlatinal hemolytic streptococci

Rabbit 6 (1,800 grams)

TOXIN STRAINS (1.1)	SKIN REACTIONS				
	August 2 (1:1)		August 18 (1.1)	August 24 (1:1)	September 10 (1:1)
Scarlatinal 1.....................	−	August 8, 10 cc.	−	++	++
Scarlatinal 2.....................	−	of toxin of scar-	−	++	++
Puerperal........................	−	latinal strain 1	−	++	++
Erysipelas.......................	−	subcutane-	−	++	++
Plain broth......................	−	ously injected	−	±	±

(−) only traumatic reaction.

(±) reddened area of diameter smaller than 0.5 cm.

(++) reddened area of diameter over 2 cm. with swelling.

inoculation of rabbits with strains from various sources, no distinct difference could be shown by Parish and Okell (1928).

RASH DEVELOPING PROPERTY

As above stated, every reaction available having failed distinctly to differentiate scarlatinal hemolytic streptococci, the only experimental criterion left of the identity of a scarlatinal strain might appear to depend on the demonstration of its specific rash-developing property when the toxin is injected into an appropriate person. Dick and Dick's statement that toxins of scarlatinal hemolytic streptococci develop a scarlatini-form rash when injected into susceptible persons, gave a scientific ground

for the observation which Gabritschewsky made when he performed his active immunization on children. I myself observed fairly well defined scarlatini-form rashes in 2.9 per cent among 1277 children of one group, and in 8.7 per cent among 3150 children of the other group, both groups received from 500 to 20,000 s.t.d. of toxin of scarlatinal strain, at the time of the initial dose.

It is as yet entirely uncertain whether toxin of non-scarlatinal origin may also develop a rash, and in case a rash develops what is the difference between it and a rash caused by the toxin of a scarlatinal strain. Langowoi (1906) vaccinated 20 children with a vaccine made from a broth culture of erysipelas origin; none of these developed a rash, whereas 120 children inoculated with a vaccine made in the same way from scarlatinal strain developed rash in 13.3 per cent, which was followed by desquamation. On the same ground, Gabritschewsky (1906; 1907) stated that the streptococcal etiology of scarlatina had a decisive proof. To throw light on this point, I injected intramuscularly in a series of children toxins of hemolytic streptococci not associated with scarlatina, strains isolated from gangrene, erysipelas, puerperal fever, and from healthy throats. The results are summarized in table 6.

Scarlatinal toxin could give rise to a rash in every instance when 0.1 to 0.5 cc. of the original toxin was injected while a large number of cases injected with non-scarlatinal did not.

The rash developing property is not, however, specific to scarlatinal streptococci because toxin of erysipelas origin also developed a rash.

Rash caused by injection of toxin of erysipelas origin was blanched with sera of convalescent patients and by antitoxic streptococci horse serum prepared against scarlatina toxin as well as by that prepared against erysipelas toxin, just as was the rash caused by injection of scarlatinal toxin, as shown in table 6.

The rash thus caused is therefore identical so far as can be judged by Schultz-Charlton's blanching phenomenon. In this respect, toxins of strains, from scarlatina and erysipelas at least, have no absolute difference.

TABLE 6

Rash developing property of non-scarlatinal streptococci toxin

NAME	AGE	SKIN REACTION		TOXIN INJECTED		RASH	BLANCHING PHENOMENON		
		Standard 1 s.t.d.	Toxin used in injection	Source	Amount (1:1)		Convalescent serum	Scarlatinal antitoxin	Erysipelas antitoxin
Oh.	8	++		Scarlatina	1.0	+	+	+	+
Ya.	8	+		Scarlatina	0.6	+	+	+	+
Ue.	7	++		Scarlatina	0.4	+	+	+	
Ku.	11	+		Scarlatina	0.5	+	+	+	
Ao.	9	+		Scarlatina	0.5	+			
Sh.	11	+		Scarlatina	0.1	+			
Te.	8	+++		Scarlatina	0.1	+			
Is.	11	++		Scarlatina	0.1	+			
Za.	11	++		Scarlatina	0.1	+			
Mi.	11	+		Scarlatina	0.1	+			
Ya.	11	+		Scarlatina	0.1	+			
Nak.	2	++	++	Erysipelas	0.5	—			
Nam.	8	++	++	Erysipelas	0 5	—			
Suf.	1	+	++	Erysipelas	0.5	+	+	+	+
Ikc.	7	+	+	Erysipelas	0.5	—			
Ta.	3	++	+	Erysipelas	0 5	—			
Oh.	16	++	+	Erysipelas	0.5	—			
Hio.	8	+	+	Erysipelas	0.5	—			
Ha.	8	+	+	Erysipelas	0.7	—			
Hat.	5	+	+	Erysipelas	0.5	—			
Ikm.	2	+	+	Puerperal	0.7	—			
Ikh.	5	+	+	Puerperal	0.5	—			
Tam.	9	+	+	Puerperal	0.7	—			
Hir.	8	+	+	Puerperal	0.7	—			
Har.	8	+	+	Puerperal	0.7	—			
Has.	5	+	+	Puerperal	0.5	—			
Ha.	6	++	++	Gangrene	0.5	—			
Ai.	2	++	++	Gangrene	0.5	—			
Iis.	3	+	++	Gangrene	0.5	—			
Tu.	1	+	++	Healthy throat	0.5	—			
An.	4	+	+	Healthy throat	1.0	—			

Original toxin was intramuscularly applicated.

In testing blanching phenomenon, 0.1 cc. of serum was intradermally injected on the breast. The intradermal injection was done prior to the intramuscular injection or at the same time with the latter.

Well defined blanching was defined as positive.

DISCUSSION

In no respect has an absolute specificity of scarlatinal hemolytic streptococci been established.   Every biologic as well as antigenic property tested seems common to a certain extent among strains of hemolytic streptococci from various sources.   Especially, it is worth while to note that rash-developing property is not limited to scarlatinal strains.   These facts superficially seem to show that hemolytic streptococci primarily have no relation with the etiology of scarlatina, as was believed by Jochmann and others.   But the fact, on the other hand, that filtrate of broth culture of scarlatinal hemolytic streptococci contain scarlatinal antigen is well established, as shown by their rash developing property and Schultz-Charlton's blanching phenomenon.   Hemolytic streptococci from other sources also produce such an antigen.   Therefore, the presence of a special filtrable virus of scarlatina may be doubted simply because the presence of such special virus, if any, together with streptococci from other sources than scarlatina is difficult to understand.   Rash-developing power as well as the property of evoking Schultz-Charlton's phenomenon are properties of scarlatinal hemolytic streptococci, but these properties are common to hemolytic streptococci from other sources though in quantitatively less degree.   Is there an unknown virus which makes hemolytic streptococci active?   This is another problem. Finally the question arises as to what is the nature of scarlatinal symptoms; are they toxic or allergic in relation to the product of the scarlatinal strain?   The question has to be decided by further experimental studies.

SUMMARY

1. By means of agglutination, precipitation and complement fixation with convalescent scarlatinal sera, scarlatinal hemolytic streptococci have not been differentiated from non-scarlatinal strains.

2. Toxin of non-scarlatinal hemolytic streptococci also produce skin reactions in a dilution as high as 1 in 1,000.

3. Toxin of non-scarlatinal hemolytic streptococci can be neu-

tralized by scarlatinal convalescent sera though with less constancy.

4. Immunization with non-scarlatinal hemolytic streptococci can give rise to blanching phenomenon.

5. Antitoxic sera against erysipelas hemolytic streptococci exerts similar effects on scarlatina.

6. A rabbit previously treated with scarlatinal streptococci may give positive skin reactions to the toxin of scarlatinal hemolytic streptococci as well as to that of non-scarlatinal hemolytic streptococci.

7. An area of human skin previously treated with toxin of non-scarlatinal hemolytic streptococci can exert a local allergic or immunity reaction when the individual later contracts scarlatina, as it is the case in an area treated with toxin of scarlatinal strain.

8. Injections of toxins of non-scarlatinal hemolytic streptococci in a positive reactor can turn Dick's skin reaction to negative.

9. Toxin of erysipelas streptococci may also develop a rash when injected in a certain amount in appropriate persons.

10. The rash caused by toxin of hemolytic streptococci of scarlatinal as well as non-scarlatinal origin is identical.

11. Scarlatinal hemolytic streptococci are those strains of hemolytic streptococci which possess a stronger tendency to develop rash in the human subject, and also stronger antigens for the blanching phenomenon.

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QUANTITATIVE ASPECTS OF THE METABOLISM OF ANAEROBES

IV. THE NATURE OF THE VOLATILE ACID PRODUCED BY C. HISTOLYTICUM FROM PROTEINS

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In a study of the biochemistry of *C. histolyticum* in cooked meat media it was noted that the volatile acid produced by this organism was practically pure acetic acid. Previous results (Parsons and Sturges, 1927) obtained on meat cultures of various anaerobes had indicated that rather complex mixtures of volatile acids were always obtained from the more common organisms. Wagner, Dozier and Meyer (1924) state that from one meat culture of *C. tetani* they obtained practically pure acetic acid. This seems to have been an exceptional instance, however, since the data they present for both sixty-hour and eighteen-day cultures of *C. tetani* are interpreted as indicating the presence of valeric, butyric, and acetic acids. Since, with the possible exception of *C. tetani*, no proteolytic anaerobe has been reported as forming a single pure acid from protein, a systematic study was made of the nature of the acid produced by *C. histolyticum* at various ages when grown on different media.

The cultures used in this study were obtained from three different sources and are designated

1. *C. histolyticum* Lister Institute
2. *C. histolyticum* Weinberg
3. *C. histolyticum* Hall

The Lister Institute strain, obtained from Reddish, yielded other species besides *C. histolyticum* on plating. Strains of the latter, isolated from the mixture, lost the sugar fermenting ability

and putrid odor reported by Reddish and Rettger (1924) for this strain, and conformed in all essential characteristics, including typical rapid digestion of muscle tissue *in vivo*, with *C. histolyticum* strains from the other sources.

C. histolyticum. Weinberg, was obtained directly from the Pasteur Institute through the kindness of Professor Weinberg.

C. histolyticum, Hall, was obtained directly from I. C. Hall who isolated it from the feces of a prisoner in Saint Quentin.

TABLE 1

Volatile acid produced for Duclaux titrations in various media

MEDIUM	TOTAL NUMBER OF DETER- MINATIONS	NUMBER OF STRAINS	AGE RANGE		VOLATILE ACID RANGE CC N/10 PER 100 GRAMS MEDIUM*	
			Mini- mum	Maxi- mum	Mini- mum	Maxi- mum
			hours	*days*		
Buffered pork	14	3	18	55	190	635
Peptone....................	12	3	18	27	75	182
Gelatin	21	3	18	27	128	520
			days			
Brain	2	2	60	60	217	242
Beef heart.....	3	3	60	60	423	440
Liver	2	2	60	60	238	293
Milk	5	3	6	15	26	69
Total.........................	59					

* Per 100 grams tissue in the case of pork, brain, heart, and liver.

The cultures were grown under suitable conditions in the following media: buffered pork, gelatin, peptone, brain, beef heart, liver, and milk. Table 1 gives the data on the number of strains used, the ranges of age studied, the total number of determinations made, and the amounts of volatile acids produced in the various sugar-free media.

The organisms grew well in all media as evidenced by the figures for volatile acids.

All Duclaux determinations were made according to the procedure previously described by the authors (1927). The

detailed data for volatile acid titration sequences in various media at various ages are presented in tables 2 to 6.

TABLE 2

Volatile acid produced by C. histolyticum in 10 per cent gelatin in hydrogen

STRAIN	AGE OF CULTURE	VOLATILE ACID, CC. N/10 PER 100 CC.	TITRATION SEQUENCES								
			10	20	30	40	50	60	70	80	90
	hours										
Lister	18	164	8 3	16 6	24 9	33 7	43 0	52 3	62 7	73 6	85.8
Lister	18	128	8 1	16 3	24 6	33 4	42 7	52 3	62 6	73 5	86.0
Hall	18	208	8 0	16 2	24.9	33 2	42 2	51 9	62.3	73.2	85.9
	days										
Lister	3	397	8 0	16.4	24.8	33 5	42 7	52.3	62 6	73 6	85.7
Lister	4	455	8.1	16 2	24 5	33 5	42 7	52 5	62 8	73.8	86.0
Hall..	4	466	8.0	16 3	24 8	33 5	42 7	52 2	62.4	73 4	85 7
Lister	6	478	8.0	16 3	24 8	33 7	43 0	52 7	62 9	73 9	85 9
Lister	8	507	8.1	16.6	25 0	33.8	42 9	52.4	62 6	73 6	85.8
Hall	8	506	8.1	16 4	24 7	33 6	42 7	52 3	62 6	73 5	85 8
Lister	12	472	8 0	16 3	24.8	33 4	42.6	52 2	62 4	73 5	85 7
Lister	20	480	8 4	16 7	24 9	33 8	42.9	52 6	62.8	73 7	86 0

TABLE 3

Volatile acids produced by C. histolyticum in gelatin in vacuo

STRAIN	AGE OF CULTURE	VOLATILE ACID, CC. N/10 PER 100 CC.	TITRATION SEQUENCES								
			10	20	30	40	50	60	70	80	90
	days										
Hall	2	367	8 6	17 3	25.8	34 2	43 1	52.7	62 5	73.5	85.6
Lister	2	318	8.2	16.1	24.7	33.5	42 5	51.9	62 0	73 2	85.4
Weinberg	2	470	8 0	16.2	24.7	33.5	42 5	51 8	62 3	73.5	85.7
Lister	4	296	8.1	16 2	24.5	33.4	42 2	51.7	61 9	73.1	85.3
Weinberg	5	520	8 2	16.5	24 8	33.7	42.8	52 4	62.7	73 6	85 8
Lister	9	340	8.0	16.0	24.5	33.4	42.2	51 9	62.1	73 2	85.6
Hall	9	443	8.4	16 9	25 4	34.1	43 2	52 9	63.0	74 0	86.0
Weinberg	13	520	8.1	16.4	24 7	33.6	42 7	52.3	62.6	73.6	85.8
Lister	15	364	8.0	16.1	24 3	33.0	42.1	51.8	62.0	73.0	85.4
Lister	27	404	8.1	16.5	24.8	33.6	42.8	52 4	62.8	73 9	86.1

Table 2 represents determinations made on cultures in 10 per cent nutrient gelatin pH 7.5, incubated in a hydrogen filled vessel at 37°C. for the periods noted.

TABLE 4

Volatile acids produced by C. histolyticum in peptone in hydrogen

STRAIN	AGE OF CULTURE	VOLATILE ACID, CC. N/10 PER 100 CC.	TITRATION SEQUENCES								
			10	20	30	40	50	60	70	80	90
	hours										
Hall	18	75	8.8	17.3	25.7	34.3	43.1	52 4	62 6	73.5	85.7
Lister	18	91	8.8	17.3	25.7	34 3	43.1	52.4	62.6	73 5	85.7
	days										
Lister	2	86	8.6	17.3	25.8	34.5	43.6	53.1	63 0	74.0	85.8
Weinberg	2	72	8.5	16.6	25.3	33.8	43.5	52 7	62 8	72 9	85.8
Hall	3	145	8 3	16.5	25 0	33 7	42.6	52 4	62 7	73.5	85.6
Lister	3	146	8 3	16 4	25.0	33.6	42 6	52 3	62 7	73.5	85.7
Lister	4	159	7.7	15 9	24 4	33 2	42 3	52.0	62 3	73.3	85 5
Weinberg	5	110	8 3	16 4	24 9	33.6	42 9	52 5	62 6	73.8	85.8
Lister..	9	176	8 0	16.3	24.6	33.6	42 6	52 3	62 5	73.5	85.8
Weinberg	13	126	8 1	16 5	25.1	34 0	43 2	52 8	63 0	73.8	86 0
Lister.....	15	178	8 1	16 2	24 9	33 6	42.7	52 4	62.4	73 5	85.7
Lister.	27	182	8 2	16 4	24 9	33 6	43 0	52 6	62 8	73.6	85.8

TABLE 5

Volatile acids produced by C. histolyticum in meat medium

STRAIN	AGE OF CULTURE	VOLATILE ACID, CC N/10 PER 100 CC.	TITRATION SEQUENCES								
			10	20	30	40	50	60	70	80	90
	hours										
Lister	18	190	8 3	16 5	25 0	33 7	42 9	52 4	62.5	74 0	85.4
	days										
Weinberg	2	315	8.5	16.5	25 0	33 8	43.1	52.6	62 9	73 9	86.1
Lister.....	3	375	8 0	16.5	24 9	33.8	43 0	52 7	62 7	73.7	85.9
Weinberg	5	470	8 1	16.4	24.9	33 8	43 0	52 7	63.0	73 9	86.0
Lister.....	6	415	8 0	16 3	24 8	33.6	43.0	52.7	63 0	73 9	86 0
Hall..	6	493	8.1	16 4	24 9	33 6	42 8	52 4	62 5	73 7	85.9
Lister.................	6	488	8 1	16.1	24.7	33.5	42 3	51 9	62 2	73 0	85.4
Hall......	9	425	8 0	16 3	24 8	33.8	42 8	52 4	62.8	73 6	86.0
Lister	12	455	8.2	16 5	25 0	33 9	42.9	52.6	62.8	73 9	86.0
Lister	20	560	8 0	16.2	24 7	33.4	42.6	52 2	62.4	73.6	85.7
Lister............. ...	20	615	7.8	16 0	24 3	32 9	42 2	51 7	61.8	72 8	85.5
Lister	20	635	8 1	16 5	24 7	33.3	42 5	51 9	62 3	73.3	85.5
Hall	20	592	8 3	16.6	25.0	33 6	42.8	52 4	62.7	73 6	85 8
Weinberg..............	55	485	8.3	16.7	25 3	34 0	43.7	52 6	62 8	73.8	86.0

TABLE 6

Volatile acids produced by C. histolyticum in miscellaneous media

STRAIN AND MEDIUM	AGE OF CULTURE	VOLATILE ACID, CC. N/10 PER 100 CC.	TITRATION SEQUENCES								
			10	20	30	40	50	60	70	80	90
	days										
Milk:											
Lister	6	58	7.8	15 9	24 2	32.8	41 9	51.4	61.6	72.7	85.1
Hall	8	65	7 9	15 9	24 2	32.7	41.8	51.3	61.7	72 6	85 1
Weinberg	15	26	7 8	16 4	24 5	33 4	42 3	52 0	62 1	72.8	85 4
Lister	15	64	8 3	16 7	25 3	33.9	43 0	52 4	62 6	73 5	85.8
Hall	15	69	7.9	16.1	24 5	33 2	42 2	51 9	62.2	73 1	86.0
Heart:											
Lister	60	423	8 0	16.4	24.8	33 6	42 8	52 4	62 6	73 7	85.8
Hall	60	440	8.2	16 5	25 0	33 6	42 8	52 4	62 7	73 6	86 0
Weinberg	60	432	8.2	16.4	24 8	33 6	42 6	52.1	62 4	73.5	85.7
Brain:											
Lister	60	217	8 2	16 6	25 1	33 9	43 1	52 6	62 8	73.7	85 9
Hall	60	242	8 4	16.7	25 4	34 2	43.4	52 9	63 2	74 2	86 3
Liver:											
Hall	60	293	8.2	16 4	24 9	33 6	42.7	52 2	62 4	73 4	85 8
Weinberg	60	238	8 2	16 6	25 2	33 9	43.1	52 6	63 0	73 8	86 2

TABLE 7

Summary of volatile acid data for all media

	TITRATION SEQUENCES								
	10	20	30	40	50	60	70	80	90
Maxima	8 8	17.3	25 7	34 5	43 7	53.1	62 0	74 0	86 1
Minima	7 7	15.9	24 2	32 7	41.8	51 3	63 0	72 6	85 1
Average	8 2	16 4	24 9	33 6	42 8	52 3	62 5	73 5	85 8
Acetic acid—pure	7 9	16 0	24.6	33.1	42 3	51 9	62 2	73 4	85 7
Deviation of average from acetic	+0 3	+0.3	+0 3	+0 5	+0 5	+0.4	+0 3	+0 1	+0.1
Mixture of acetic acid 96 per cent and propionic 4 per cent	8.1	16 3	25.0	33.6	42 8	52 4	62.7	72 8	86 0
Deviation of average from mixture	+0.1	+0.1	-0.1	0 0	0 0	-0.1	-0 2	-0.3	-0.2

Table 3 represents determinations on cultures grown in 10 per cent nutrient gelatin, pH 7.5 in sealed evacuated glass containers.

The cultures listed in table 4 were grown in 5 per cent peptone medium adjusted to pH 7.5 and incubated in hydrogen at 37°C. for the specified times.

The material for the fourteen Duclaux determinations shown in table 5 was obtained by extracting buffered pork cultures of *C. histolyticum* of a wide range of ages.

Table 6 shows how consistent the action of *C. histolyticum* is on such widely differing proteins as those of milk, beef heart, brain, and liver.

Preliminary investigation has failed to give any evidence of the production of non-volatile acids by *C. histolyticum*. Analyses of cultures in several media show agreement of volatile acid and total acid figures to within 1 per cent. Direct determinations for lactic acid by the method of Friedmann, Cotonio, and Shaffer (1927) also gave negative results.

DISCUSSION AND CONCLUSIONS

In order more readily to evaluate the results, all the data have been analyzed in table 7. This table shows that the averages of the 59 Duclaux determinations and the values determined for pure acetic acid with the same apparatus are almost identical. It may be seen that the deviations are little more than might be expected from experimental errors. The figures designated maxima do not represent a single sequence but are composited from the highest of the 59 values for each of the 10 cc. fractions. The minima are similarly composited from the lowest values ever obtained for each fraction.

The slight positive deviations of the average results from pure acetic acid figures are such as might result from the presence of slight amounts of some higher volatile acid. An assumption of 96 per cent acetic-4 per cent propionic is made in the table not as a satisfactory analysis of the data (since the deviations show a definite trend from plus to minus) but merely to show the *maximum possible* admixture of a higher volatile acid and to indicate how well justified we are in considering the histolyticus product as pure acetic acid. If the presence of any volatile acid higher than propionic were postulated a still smaller amount would be allowed by the Duclaux values.

That there was present no appreciable amount of formic acid was demonstrated in the following manner. The still residues, in which formic acid if present would tend to concentrate, were collected from several Duclaux runs. These were combined and
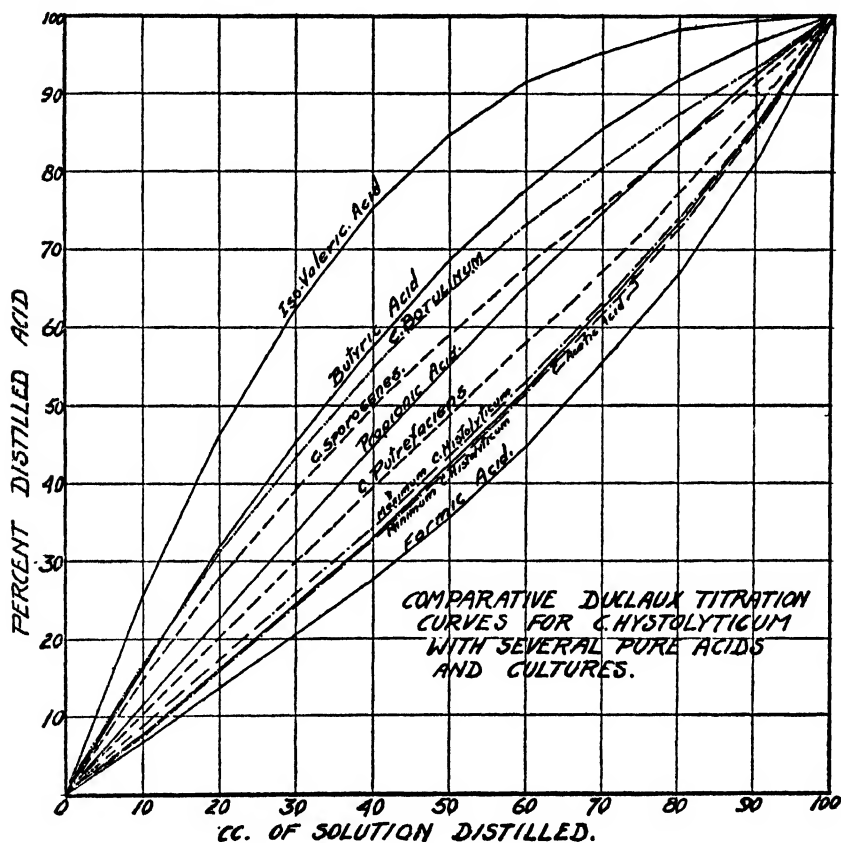

CHART 1

in turn subjected to a Duclaux distillation. The values obtained were in close agreement for acetic acid.

The property of yielding from proteins the single volatile acid—acetic—is, so far as our observations go, peculiar to *C. histolyticum*. The other putrefactive anaerobes studied always produce mixtures of two or three volatile acids from proteins.

Chart 1 combines in graphic form the Duclaux data presented in this article with data previously published by Parsons and Sturges (1927). The close coincidence between the curves for acetic acid and *C. histolyticum* cultures, as compared with the nearest curve for any other anaerobe (*C. putrefaciens*) is very striking. The curves for all other anaerobes studied fall farther from the acetic curve than does that for *C. putrefaciens*.

It is noteworthy that when *C. histolyticum* hydrolyzes proteins to amino acids, and splits these amino acids into ammonia and

TABLE 8

Glycine available in different protein media

	PROTEIN SOURCE	
	Gelatin Medium	Pork Tissue
Nitrogen, per 100 grams source....	1 65 grams*	2 95 grams†
Protein, per 100 grams source (N × 6.25)... .	10 30 grams	18 50 grams
Glycine, per 100 grams protein	25 50 grams‡	2.10 grams§
Glycine, per 100 grams source	2 64 grams	0.39 grams
Acetic acid from glycine, per 100 grams source (60 glycine ÷ 75)	2 11 grams	0 31 grams
Same, expressed as cc. of N/10 acetic acid per 100 grams source	352 00 cc.	51.67 cc.
Acetic acid actually found (table 1) maximum	520 00 cc.	635.00 cc.

* Sturges and Parsons (1926).

† Parsons and Sturges (1927, p. 181).

‡ The analysis of Dakin (1920) is used because it gives the highest glycine figure for gelatin.

§ In absence of analyses for pork muscle, figures given by Osborne and Jones (1909) for ox muscle are used.

organic acids, acetic is the only acid produced regardless of the protein acted upon. This might readily be explained by the hypothesis that glycine (amino-acetic acid) is the only amino acid deaminized by this anaerobe, but there is insufficient glycine in some of these proteins to account for the amount of acetic acid produced. Computations showing this are presented in table 8.

In an attempt to shed light on the mechanism of this proteolysis a preliminary study has been made of the gas metabolism of *C. histolyticum*. Cultures were grown in buffered pork in glass

bombs thrice evacuated to the vapor pressure of water and sealed *in vacuo.* After the periods of incubation indicated, the bombs were connected to a Toepler pump, cracked open and the contents acidified and the gases completely removed and analyzed.

From these data it appears that the facts to be accounted for are the presence, in the end products, of carbon dioxide, ammonia, and acetic acid in the molar ratios of 1:2:2. Can these be accounted for by the usual text book conceptions? Consulting Waksman's "Principles of Soil Bacteriology" we glean the following possible mechanisms for the anaerobic production of CO_2:

1. Decarboxylation of di-carboxylic amino acids preceeded or followed by reductive deaminization, yielding NH_3, CO_2, and volatile acid.

$$\overset{2H}{HOOC \cdot CH_2 \cdot CH \cdot NH_2 COOH \rightarrow CH_3 \cdot CH_2 \cdot COOH + CO_2 + NH_3}$$
$$\text{Aspartic acid} \qquad\qquad \text{Propionic acid}$$
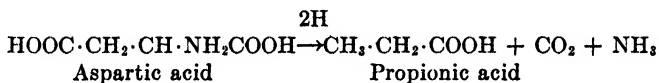
2. Decarboxylation of mono-carboxylic acid, yielding CO_2 and an amine or alcohol. The former is said (Rettger, 1912) to be characteristic of certain so-called putrefactive processes.

$$R \cdot CH \cdot NH_2 \cdot COOH = R \cdot CH_2 \cdot NH_2 + CO_2$$
$$R \cdot CH_2 \cdot NH_2 + H_2O = R \cdot CH_2OH + NH_3$$
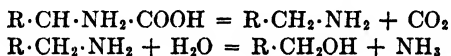
3. Degradation of chains by oxidative procedures. This assumes that oxygen or its equavalent becomes available from some oxidation-reduction system brought about by the organism. It has been postulated by Quastel and associates (1925) that such systems furnish the basis for anaerobic growth. They (1926) and independently Hosoya (1925) demonstrated that cysteine hydrochloride acting as a hydrogen donator induces growth of *C. sporogenes* in a casein digest broth in an open tube.

The inadequacy of these type reactions to account for the observed facts can best be appreciated if we consider them in order:

1. Aspartic acid yields propionic acid, CO_2, and NH_3 in the molar ratios of 1:1:1. Glutamic acid would give the same, with butyric taking the place of propionic acid. There would seem to be no dicarboxylic amino acid which would yield acetic acid

without further decarboxylation, and the ratio $CO_2:NH_3$ is already too high to fit the culture findings.

2. The objection to this reaction is somewhat the same. While acetic acid could presumably be thus produced by oxidation of the alcohol it must always be accompanied by at least equivalents of CO_2 and of NH_3, thus giving too high a $CO_2:V.A.$

3. Even admitting the possibility of the presence in sugar-free meat and gelatin media of some suitable oxygen source, the degradation of long chains to the acetic stage would be untenable. Each molecule of acetic acid thus derived must be accompanied by at least one molecule of CO_2. The ratio $CO_2:V.A.$ in the end products would then have to be unity or greater.

We have already shown (table 8) that according to the data there is present in meat only sufficient glycine to account for about one-twelfth of the acetic acid produced by *C. histolyticum*. It is now evident that if any considerable portion of it resulted from any of the reactions listed above we would have vastly greater quantities of CO_2 produced than we were ever able to find analytically.

Hence, we are forced to conclude either

1. That this organism and possibly all putrefactive anaerobes have some highly specific method of splitting proteins, differing radically from the usually accepted hydrolysis, or

2. That *C. histolyticum* has an unprecedented method of splitting long chain amino acids into acetic acid and residues (amines, alcohols, etc.) other than CO_2 which thus far have not received due recognition.

It is to be hoped that further quantitative studies will clear up these questions and give us some definite conception of the mechanism of proteolysis, that most important phase of the metabolism of the anaerobes. For such studies *C. histolyticum* seems especially adapted. It hydrolyzes a variety of proteins rapidly with the production of large amounts of NH_3 and the single volatile acid-acetic—and the absence of highly putrefactive odors indicates a minimum of organic sulphur decomposition products.

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THE INFLUENCE OF AZOTOBACTER CHROOCOCCUM UPON THE PHYSIOLOGICAL ACTIVITIES OF CELLULOSE DESTROYERS

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INTRODUCTION

The stimulative effect of Azotobacter on cellulose destroyers in association studies has been previously observed by one of us (Sanborn, 1926). It was shown that increases in growth and physiological efficiency on the part of the latter organisms were obtained in association with living Azotobacter cells. The interpretation of this phenomenon was considered similar to the one given by Bovie (1924) for the stimulative responses of amoebae in a "radiation environment." Azotobacter cells appeared to produce a stimulation environment in the medium which affected other species of bacteria coming within its sphere of influence.

The present investigation substantiates the conclusions drawn from this initial study and offers a possible explanation for the phenomenon.

EXPERIMENTAL

The first of the two species used in this work comes nearest to the description given for *Cellulomonas subcreta* (McBeth) but differs in the sugar reactions. This organism answers the following description (Hamilton):[1]

Gram-negative, motile rod with polar flagella; non-spore-forming, probably facultatively anaerobic; gelatin not liquefied; indol not

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[1] Hamilton, W. B. Gum production by *Azotobacter chroococcum* Beijerinck and its physiological significance. M.Sc. thesis, McGill University, 1928.

formed; milk unchanged; acid and gas formed in glucose and fructose broth, and a slight amount of acid in xylose and glycerol; decomposes raw cotton in the China blue-aurin medium (Sanborn 1927).

Cellulomonas folia,[2] (Sanborn, 1926), also used in the investigation, has been described as follows:

Gram-negative, small rods (1.0–1.5 by 0.8–1.0 microns), motile by means of peritrichous flagella numbering two to six; non-spore-forming; facultative anaerobic; gelatin slowly liquefied; sugars not fermented, starch hydrolyzed; NH₃ produced; litmus milk alkaline with slight peptonization; indol not formed; H₂S not formed; nitrates reduced to nitrites.

The stimulative influence of *Azotobacter chroococcum* upon the rate of cellulose fermentation, noted in the previous work, has been confirmed in a recent investigation (Hamilton, 1928) for the organisms mentioned above. Using the China-blue-aurin-cellulose reaction as an index to physiological efficiency it was found that during the time of the experiment, four to eight days, the cellulose destroyers were unable to set up active fermentations; in association with living Azotobacter cells, however, the fermentations of cellulose were well under way in two days.

In searching for an explanation of this accessory action, attention was directed to the gum produced by Azotobacter as a possible source of an essential food substance for cellulose destroyers. Reference has been made previously to the slime formed by this organism (Stapp, 1924; Heinze, 1926). Growth promoting qualities have also been associated with its activities (Hunter, 1923; Bottomley, 1920; Mockeridge, 1924). In the present work *Azotobacter chroococcum*, isolated from soil in this locality, was employed. Its development in various solutions was investigated, using numerous carbohydrates. These were present in 2 per cent concentrations. The media were inoculated with Azotobacter suspensions adjusted to a turbidity of ten according to the McFarland scale. The cultures were incubated at 30 degrees for sixteen days. The gum was precipitated and purified by the method suggested by Buchanan (1909).

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[2] The complete description of this organism corresponds most closely to that given for *Cellulomonas rossica* (Kellerman) Bergey et al.

Using various carbohydrate sources it was found that approximately the same amounts of gum were produced under similar experimental conditions. Dextrin and inulin, however, yielded more gum than did any of the other carbohydrates, although a peptone-sucrose mixture gave good results.

TABLE 1

TIME	CELLULOSE DESTRUCTION BY CELLULOMONAS SP.		CELLULOSE DESTRUCTION BY CELLULOMONAS IN PRESENCE OF AZOTOBACTER	
	Series I	Series II	Series I	Series II
days				
1	—	—	—	—
2	—	—	++	++
3	—	—	++	++
4	—	—	++	++
7	—	—	++R	++R
8	—	—	+R	+R

TABLE 2

TIME	CELLULOSE DECOMPOSITION BY CELLULOMONAS FOLIA ALONE		CELLULOSE DECOMPOSITION BY CELLULOMONAS FOLIA IN ASSOCIATION WITH AZOTOBACTER	
days				
1	—	—	—	—
2	—	—	++	++
3	—	—	+R	+R
4	—	—	+R	+R

—, no change; no visible alteration of cellulose. +, blue color appears at surface of medium; cellulose layer begins to decompose. ++, supernatant layer is bright blue and color extends into the cellulose layer, particles of cellulose show definite flocculation into large clumps. +++, entire medium becomes of an intense blue; cellulose rapidly dissolves leaving small residue of undecomposed material. R, reduction of indicator.

The gum is similar chemically to that produced by Rhizobium and described by Buchanan (1909). The Azotobacter gum differs apparently in being precipitated by a normal solution of neutral lead acetate. The product was identified as of the "arabin" type. It is a carbohydrate, laevo-rotatory, and is not readily hydrolyzed by boiling with acid. The micro-Kjeldahl test showed a trace of combined nitrogen.

In order to test the accessory effect of this gum the China-blue-aurin method was used, adding varying amounts of gum from a sterile stock solution containing 6.60 mgm. of gum in 1.0 cc. of distilled water. Approximately 900,000 organisms of the unidentified Cellulomonas were added to each tube of China-blue-aurin-cellulose solution.

Table 3 merely indicates the possible accessory function of the Azotobacter gum, and is a preliminary report of a general investi-

TABLE 3

The increase in growth and physiological efficiency of a cellulose decomposer through the addition of sterile gum produced by Azotobacter chroococcum

AMOUNT OF GUM	AGE OF CULTURE					
	1 day		2 days		3 days	
	Growth	Reaction	Growth	Reaction	Growth	Reaction
cc.						
0.1	+	−	+++	++		++
	+	−	++	++		+++
0 25	+	−	+++	++	Maximum development of pellicle	++
	+	−	++	++		++
0.5	++	−	+++	++		++
	++	−	+++	++		++
1 0	+++	−	+++	+++	Surface disturbed	+++R
	++	−	+++	+++		+++
Control	−	−	++	+		++
	−	−	++	+		++

gation still in progress. Not only does this organism form considerable amounts of gum on laboratory media, but it is highly probable that this function forms part of Azotobacter activity in the soil. A morphological study of Azotobacter direct from soil indicates that under certain conditions a form of the organism appears in which the cell membrane assumes a diffluent or mucilaginous character.

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OBSERVATIONS ON SOME OF THE FACTORS INVOLVED IN FILTRATION EXPERIMENTS

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That the result of any filtration experiment is dependent on a number of different factors, that these factors must be carefully controlled and the exact conditions under which any such experiment is carried out accurately set forth is now well recognized, and has been thoroughly discussed and well summarized by Mudd (1928).

Mudd (1922) and Kramer (1928) have shown that the passage of any substance through a filter depends not only on the relation of the size of the particles in suspension to the size of the pores of the filter, but also upon the relation of the electrical charge on the filter to the electrical charge on the suspended particles. Thus any siliceous filter, such as the Berkefeld, Chamberland or Mandler, carrying a negative charge, will allow the passage of the acidic dye, Congo red, but will hold back basic Victoria blue B.

That the preliminary passage through a filter of egg white, peptone, heated serum or oil allows the subsequent passage of substances which are ordinarily held back has been demonstrated by Holderer (1912), Muir and Browning (1908–1909) and Holman (1926).

Bronfenbrenner (1927) found that if particles carrying bacteriophage were deposited on a Berkefeld filter, the bacteriophage could be washed through the filter with broth, but not with water. Ward and Tang (1929) have demonstrated that the virus of herpetic encephalitis and the virus of vaccinia are more uniformly filterable when emulsified in hormone broth, than when emulsified in normal saline.

Ten Berkefeld V filters which had been used one or more times and thoroughly cleaned were tested for the rate of flow of distilled water at 10 cm. negative pressure, the amount of pressure required to bubble air through them under water, and their permeability for young and old cultures of *B. prodigiosus*.

In each case, 5 cc. of a twenty-four-hour culture of *B. prodigiosus* were filtered under 10 cm. Hg negative pressure, and 1 cc. of the filtrate was inoculated into each of two tubes containing 5 cc. of hormone broth. One cubic centimeter of a twenty-eight-day-old culture of *B. prodigiosus* was then added to the culture in the filter, 5 cc. filtered and 2 cc. of the filtrate cultured as before.

TABLE 1

	FILTER									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Distilled water in 5 minutes at 10 cm. negative pressure, cc. .	34	31.5	32.5	59	59	48	41.5	48	69	69
Pressure required for passage of air, pounds..	9.7	5	5	9.7	10	9.7	6	7.5	5	4.5
Filtrate of 24-hour culture of *B. prodigiosus*	++	++	++	--	--	--	--	+-	--	--
Filtrate of 28-day culture of *B. prodigiosus*	++	++	++	++	++	+-	+-	++	++	+-

Each + sign means growth of *B. prodigiosus* in 1 cc. of filtrate inoculated into 5 cc. of hormone broth.

It was found, as shown in table 1, that not only did the two methods of testing the permeability of the filters not always agree, but that neither test showed whether the filter would hold back the culture. The old culture of *B. prodigiosus* came through all ten of the filters in the first 5 cc. of filtrate.

In attempting to produce a collodion filter which would separate the filterable viruses from *B. prodigiosus* without the considerable loss of virus which always occurs during Berkefeld filtration, it was found that a considerably higher percentage of collodion was necessary to hold back *B. prodigiosus* if suspended in hormone broth than if suspended in normal saline. Collodion filters were now tested for permeability to acidic and basic dyes.

In aqueous solution, Congo red came through without loss of color; but Victoria blue B was held back. However, if the Victoria blue B were made up in hormone broth, it passed through the filter with practically no loss of color.

A number of new and of used Berkefeld filters, V and N, were now tested for their permeability to Victoria blue B. Unless otherwise stated, filtration was carried out at 10 cm. Hg negative pressure, the dye made up in a 1:20,000 dilution and adjusted to pH 7.8.

The dye in distilled water or in 1 per cent phosphate buffer was always completely held back. In hormone broth the dye passed through all the filters in from one-half to full strength. If the hormone broth was given a preliminary filtration through a Berkefeld or through paper, or was autoclaved for an hour at 15 pounds, it still brought the dye through, but not so readily. In extract broth the dye filtered but slightly. Peptone solutions were treated in various ways, but none of these preparations caused the passage of the dye through the filter, and it was found that the omission of peptone from the broth increased its filtering capacity. Broth prepared by the following method was considerably more effective in allowing the filtration of Victoria blue than was the stock hormone broth.

Four hundred grams of minced beef heart per liter of water is extracted over night in the cold. Boil fifteen minutes and strain through a wire sieve. Add 0.5 per cent sodium chloride and 35 cc. $N/1$ NaOH per liter. Autoclave at 15 pounds for forty-five minutes. Allow to settle over night. Siphon off and adjust to pH 7.8. Autoclave at fifteen pounds for fifteen minutes.

The dye was found to filter much more completely if the filtration were carried out on the alkaline side of neutrality, the optimum reaction being about pH 7.8. This effect seems to be due to the more complete solubility at this point of the substance responsible for the filtration, rather than to a change in the charge on the particles of dye.

It was noticed that the ability of the various broths to carry the dye through a filter corresponded to the amount of precipitate obtained by acidifying the broth to about pH 4.6, and that this

precipitate contained the bulk of the substance responsible for the filtration.

Whole horse blood, or washed horse corpuscles autoclaved with dilute sodium hydrate will take the dye through a filter even more effectively than does the broth.

If a 10 per cent suspension of washed yeast cells in N/5 NaOH is autoclaved for thirty minutes, centrifuged and adjusted to pH 7.8, the supernatant fluid brings the dye through the filter even when diluted to one part in four hundred.

TABLE 2

	FIRST 20 CC.	SECOND 20 CC.	THIRD 20 CC.
Berkefeld V, no. 1			
Salt solution	— —	— —	— —
Hormone broth	— —	— —	— —
Broth B*	+ —	— —	+ +
Berkefeld N, no. 1			
Salt solution............................	— —	— —	— —
Hormone broth	— —	— —	— —
Broth B*................................	+ +	+ +	+ +
Berkefeld N, no. 2			
Hormone broth.	— —	— —	— —
Yeast solution	— —	+ +	+ +

* Broth B is the peptone-free broth described in the text.

From these results it seemed most probable that the source of the substance which effected the filtration of the dye was the lecithin of the meat, blood or yeast cells.   A 1 per cent suspension of lecithin in dilute sodium hydrate was, therefore, autoclaved and filtered through paper.   This filtrate in a dilution of one in a hundred was still found to bring about the filtration of the dye quite readily.

The experiments given in table 2 show the influence of the suspension fluid on the filterability of *B. prodigiosus*.   One cubic centimeter of a twenty-four-hour broth culture of *B. prodigiosus* was added to 100 cc. of the suspension fluid, and from each 20

cc. of filtrate, 3 cc. were inoculated into each of two tubes containing 5 cc. of hormone broth.

DISCUSSION

The investigation here recorded was undertaken because of the difficulties experienced by my colleagues, in working with the filterable viruses, in determining beforehand whether a given filter would prove impermeable to the bacteria used for control.

The rate of flow of distilled water obviously fails to distinguish between a moderately loose filter with uniform pores which will hold back bacteria, and a tight filter with one or more gross defects. The air pressure test, while bringing to light large leaks such as occur about the collar of the filter, is of little value in showing whether or not *B. prodigiosus* will be held back. The only adequate test, therefore, is the use at the time of the experiment of a small, easily growing organism, such as *B. prodigiosus*, as control. Here again two factors—the age of the culture and the nature of the suspension fluid—are of vital importance.

The fact that an old culture of *B. prodigiosus* passes so readily through Berkefeld V filters and that a young culture, in a suspension fluid which varies but slightly from the broth media in common use, will pass through Berkefeld V and N filters would make it seem possible that the reported filtration of such organisms as streptococci, bacilli of the enteric group, diphtheroid bacilli and tubercle bacilli might be due rather to the passage of fragments of the bacteria in old cultures still capable of reproduction or to filtration in a particularly favorable suspension fluid than to the existence of a filterable stage in the life cycle of the organism.

The nature of the substance which makes the filters more permeable is as yet undetermined. It is present in alkaline extracts of beef hearts, red blood cells, and yeast. It comes down with the precipitate formed at about pH 4.6. It is soluble in 80 per cent alcohol; but insoluble in absolute alcohol and ether. It is, therefore, not—as was at first supposed—a lipoid or soap derived from the lecithin of the cells. The determination of the chemical composition of the substance is not being undertaken, as this would require the employment of analytical methods not

within the scope of the writer's work.   It is not easy to determine whether this substance effects filtration by reversing the charge on the particles in suspension, or on the filter, or—as is more likely—by merely coating the pores of the filter in such a way that the positively charged dye does not come in contact with the negatively charged filter.   In the case of bacteria, this material may perhaps merely act as a lubricant, allowing the organisms to pass more readily through the pores.   However this may be, the influence of the nature of the suspension fluid on such experiments as aim at the determination of the size of the filterable viruses or other particles by means of filtration is of the greatest importance.

CONCLUSIONS

1. Neither the rate of flow of distilled water nor the pressure required to bubble air through a filter under water is a safe guide as to whether or not the filter will hold back bacteria.

2. An old culture of *B. prodigiosus* will pass through a filter which is impermeable to a young culture.

3. The nature of the suspension fluid is of great importance in determining whether or not particles of a given size will pass through a filter.

4. Victoria blue B will pass through Berkefeld filters, if dissolved in hormone broth.

5. Young cultures of *B. prodigiosus* will pass through Berkefeld filters V and N, if suspended in a modified hormone broth or in alkaline extracts of certain animal or vegetable cells.

6. The chemical composition of the substance present in hormone broth and in certain cell extracts which facilitates filtration through Berkefeld filters has not been determined.

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STUDIES ON DENTAL CARIES, WITH SPECIAL REFERENCE TO ACIDURIC ORGANISMS ASSOCIATED WITH THIS PROCESS

I. ISOLATION AND DESCRIPTION OF ORGANISMS

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INTRODUCTION

Though dental caries in man is known to be of almost universal occurrence, at least in the more civilized races, little has as yet been accomplished in the field of prevention. The problem is, however, receiving more and more consideration in family, school and community hygiene. Particular attention has been directed in recent years to chronic focal infection in teeth and its relation to systemic disorders of one kind or another.

Since Hippocrates proposed his "stagnation theory" of tooth decay in 466 B.C., various theories have appeared in the literature, but none have as yet met with general acceptance. The peculiar anatomical position, histological structure and chemical composition of teeth are factors which must of necessity play a large rôle in the pathological process known as tooth decay.

In more recent years a large group of investigators appear to lean toward the view that caries is a definite disease process which is induced by bacteria through the acids formed by them from carbohydrates taken in as food; that is, that initial decay is brought about by decalcification due to these acids. On the other hand, there are many who believe that the decay is primarily due to deficient diet with resulting faulty development and nourishment of the teeth, and that bacteria play only a secondary rôle. Both of these views appear to be well within the

scope of possibility; various other factors may be concerned, also, at least in a contributory way.   Investigations into the origin of dental caries must, therefore, be conducted on a broad scientific basis, in order to be at all complete.

The present investigation is being conducted chiefly from the bacteriological point of view, and as a bacteriological study has limited itself to a distinct type of bacteria known as the aciduric group.

Whether a specific type or species of organism plays an essential rôle in the etiology of caries, has long been an open question. Most of the earlier workers believed that the disease is a result of the combined action of different mouth bacteria (Miller, 1882; Goadby, 1903).   An etiological relationship of certain streptococci to the disease has been emphasized by some workers (Goadby, 1910; Kantorowicz, Baumgartner, 1913; Hartzell and Henrici, 1917; Seitz, 1921); and within the past four or five years considerable attention has been directed in some quarters to the group of aciduric organisms.

Interest in this type of bacteria was stimulated principally by the work of McIntosh and his co-workers (1922), who reported finding a specific organism, "*B. acidophilus-odontolyticus*," associated with caries.   They believed this bacterium to be different from *B. acidophilus* of Moro (1900), and claimed that it bears a definite etiological relationship to dental caries.   In the same year Rodriguez (1922), working independently, reported similar findings.

Clarke (1924) announced what he thought to be a new type, "*Streptococcus mutans*," as an important agent in tooth decay. This organism produced relatively large amounts of acid.

Hilger (1924) believed *B. necrodentalis* and *B. acidophilus* to be identical, and suggested the name *Bacillus lacticus* (Kruse) for these two organisms.

Bunting and Palmerlee (1925) came to the conclusion that the outstanding organism associated with caries is an acid-forming type resembling *B. acidophilus* of Moro, and designated it by this name.   They made no systematic taxonomic study of their isolated organism, however, and arrived at their conclusion re-

garding identity almost wholly upon morphological grounds, and as a result of limited observations. In their publication they lay much stress on the constant occurrence of their so-called "*B. acidophilus*" in decayed teeth and in the saliva of persons possessing such teeth. Their organism is apparently identical with "*B. acidophilus-odontolyticus*" of McIntosh and his associates who, like Bunting and Palmerlee, held that it bears a direct etiological relationship to dental caries. The bacillus of Rodriguez is also to be classed here.

It is apparent that the aciduric type of lactic acid bacillus has in recent years received much attention as a supposedly important factor in this pathological condition. It may be said, however, in all fairness, that the final proof of a definite etiological relationship is yet to be supplied.

A study of the prevalence of aciduric organisms in decayed teeth and in the saliva of carious mouths, and the determination of the exact position of such organisms in an accepted classification scheme, appeared to the author to be of extreme importance. The work reported here was begun in 1925.

SELECTION OF CASES

If, as has been claimed, certain aciduric organisms play an important rôle in dental caries, they should be found more or less constantly in teeth which are subject to active decay. This reasoning should apply where the pathological process is in the early, as well as in the more advanced, stages. This type of bacterium should be present also, at least with some degree of regularity, in the saliva of persons subject to progressive caries.

Another point of interest was to determine how early in the life of an individual the caries type or types of aciduric organisms occur in the mouth, and whether the individual enjoys temporary immunity at one or another period in his life.

In the present study cultures were made only from teeth in which decay was still limited to the enamel, or cases in which distinct caries cavities were not definitely apparent but in which the pathological condition was discernible by discoloration spots, or softened enamel. At the same time cultures were made from

the placques of the suspected regions and from the saliva of these early cases. Isolation tests were also made with the saliva of persons suffering from advanced caries, and of persons having caries-free mouths.

Since children appear to be particularly subject to caries, the cases were selected mainly from children ranging from about three to ten years of age. To a certain extent infants from three to eight months old were also used as subjects.

Most of the cases were supplied by the Dental Clinic of the Harvard Dental School, by the Clinic of the Forsyth Dental Infirmary for Children in Boston, and by the Baby Health Conference Clinic of the New Haven Visiting Nurses Association.

ISOLATION OF ACIDURIC ORGANISMS

At the outset cultures were made from the surface of decayed enamel by scraping with sterile swabs. For obtaining material from the deeper, decayed parts the tooth was separated from adjoining teeth and from the gingival tissue by means of a rubber dam or similar device. The enamel surface was cleansed with cotton and treated with tincture of iodine, followed by alcohol. Culture material was then procured from the isolated tooth by drilling the decayed enamel out with a sterile drill. Sterile swabs were used for making the cultures from the saliva. The materials under test were cultured by direct inoculation into 1 per cent glucose hormone or yeast broth, pH 4.8 to 4.2, and the inoculated medium incubated at 37.5°C.

After incubation for at least forty-eight hours, transfers were made to new acidified broth, and the process of incubation and transfer repeated at least two or three times at incubation intervals of twenty-four hours. About 0.5 cc. inoculum was used in each transfer. In this manner there were obtained in many instances what appeared to be pure cultures of a high-acid-forming and acid-tolerating Gram-positive organism.

To insure complete purification, the plate method of isolation was finally resorted to and cultures made from what appeared to be pure, characteristic colonies. These final cultures were subjected to glucose broth (pH 6.8) and agar plating tests, as a final

guarantee against impurities, and the isolated strains preserved as stock cultures for future study.

Only such organisms as possessed the following characteristics were retained: Gram-positive staining, production of relatively large amounts of acid or acids from carbohydrates, and unusual tolerance for acids.

Attempts were made also to isolate aciduric organisms by the use of neutral or slightly alkaline glucose media and under prolonged cultivation.

Smears made from twenty-four-hour cultures revealed many morphologically different varieties, including both Gram-positive and Gram-negative types. However, after at least four or five days incubation, the numbers of non-aciduric forms were relatively much smaller, and in some instances Gram-positive aciduric organisms appeared to predominate. The acidity of these cultures had been increased from the original, pH 6.8, to pH 4.2, and at times even to pH 3.5. The increasing acidity, of course, favored the aciduric types.

Though aciduric forms may be isolated readily by either of these two methods, the use of the acidified medium at the beginning is highly to be preferred, because it is selective all the way through. It was soon found that the direct plating method is far from being as satisfactory as the acid broth enrichment method. The microaerophilic or partially anaerobic nature of the freshly cultured aciduric organisms is without doubt an important contributing factor here.

FREQUENCY STUDY

As will be seen in table 1, high-acid forming and acid-tolerant organisms were found almost constantly in enamel caries. Among 60 individual cases under test, 93 per cent yielded positive cultures. The positive results obtained with the saliva of these subjects reached a total of 98 per cent, as compared with 17 per cent for non-carious mouths, in the few examinations made. Placques on the surface of decaying teeth revealed aciduric bacteria in every instance that tests were made.

Aciduric organisms were apparently rare in the saliva of in-

fants. In fact, no clean-cut results were obtained to show that they were present at all.

MORPHOLOGY AND STAINING PROPERTIES

The aciduric organisms isolated from teeth and saliva possess the common characteristics of the Lactobacillus genus. They are rods which vary in size more or less according to the individual strains, measuring from 0.4μ to 1.2μ in thickness and from 0.7μ to 6.0μ in length, with slightly rounded ends as a rule. Some strains appeared as slender rods having slightly tapering

TABLE 1

Showing the prevalence of aciduric organisms in carious teeth and in saliva

| | | ISOLATION | | | |
| | TOTAL CULTURES | Positive | | Negative | |
		Number	Per cent	Number	Per cent
I. Carious teeth	60	56	93	4	7
Saliva from the same cases	60	59	98	1	2
II. Surface of decayed enamel	15	15	100	0	0
III. Saliva:					
A. Carious mouth...	54	53	98	1	2
B. Non-carious mouth..............	6	1	17	5	83
IV. Saliva of infants:					
A. Breast-fed......................	19	1?	5	18	95
B. Bottle-fed.....................	12	2?	17	10	83

ends. They occurred as single cells, in pairs, or in chains. The arrangement of the cells was rather characteristic, and at times groups of them assumed a palisade-like appearance. In some instances the morphology was not very unlike that of *C. diphtheriae.* Some strains had the tendency to produce long chains, particularly in fluid media, the chains occurring in more or less tangled masses. Long threads or filamentous forms were often seen, notably in old anaerobic cultures or when the cultures were liberally supplied with carbon dioxide. In old cultures evidences of involution or degeneration forms were quite apparent, but without a tendency to real branching. The bacilli were non-motile, non-sporulating and non-capsulated.

The organisms stained readily with the ordinary anilin dyes. In young cultures they were Gram-positive, but this property was lost to a large degree as the cultures grew older. When stained by the Neisser method or by Loeffler's methylene blue, some strains showed granules while others took a uniform stain. The granule appearance was not constant, however.

The following distinct morphological groups were observed:

Group I. Very small, short straight or curved rods usually occurring in masses or clumps. They tended to form chains. Their Gram-staining property was usually less strong than that of the other three groups.

Group II. Medium-sized, straight or slightly curved rods. Some strains were rather slender with slightly tapering ends. At times they occurred in clusters. The Gram-straining property was medium to strong.

Group III. Short, thick rods, as a rule. Frequently long chains of strepto-bacilli and medium to long thread-like forms were present in the cultures. This type was more or less pleomorphic. The Gram-staining reaction was strongly positive.

Group IV. Medium to thick rods of varying lengths, often arranged in clusters. The Gram-staining ability was pronounced. The bacilli often assumed a granular appearance.

VARIATION IN MORPHOLOGY

Bunting and Palmerlee ascribed pronounced pleomorphism to the organisms isolated by them from carious teeth and from saliva. They were not in sympathy with Clarke's claim that his coccoid form, "*Strept. mutans*" is a distinct species. However, they did not make direct comparisons of this type or "mutant" with their own aciduric organisms. Furthermore, their own observations of pleomorphism were made apparently on old cultures.

In the present studies the thickness of the individual cells was more or less constant in young cultures, while the length varied, often between wide limits. In old cultures variation was readily observed. An acid reaction appeared to be more favorable for variation than an alkaline one.

Short, straight bacilli were often found to assume ring and horse shoe shapes. Strepto-bacillary forms occurring in distinct chains were frequently met with in broth cultures, whereas distinct rods appearing in clumps or huge clusters were usually found on solid media. Long, tangled, irregular-staining filamentous forms were also frequently present in old anaerobic cultures, but much less commonly in young aerobic cultures. Almost all morphological forms were seen in old cultures.

The frequency and degree of variation varied according to the different individual strains. Manifestations of certain particular forms were almost always met with in certain cultures and under certain conditions. However, the writer has been led to conclude from his own observations that the aciduric organisms associated with dental caries are not highly pleomorphic, when observed in young cultures grown under the usual conditions.

GRANULE FORMATION

On various occasions aciduric organisms of the mouth exhibited a marked similarity to members of the Corynebacterium group in the form and grouping of cells, and in the appearance of metachromatic granules. This morphological resemblance assumes some importance from the standpoint of diphtheria diagnosis by the routine method so generally adopted.

Luessen and Kühn (1908) and White and Avery (1910) described two general types of *B. bulgaricus* which they distinguished from each other largely on the basis of granule appearance in the cells. Kuntze (1908) believed that the presence of granules is not a valuable point of differentiation, since it is not constant or permanent. Cruickshank (1925) claimed that granule formation in *B. bifidus* is not a definite characteristic.

In the present work 12 strains of aciduric organisms, not including *B. bulgaricus* and *B. bifidus*, were employed. Twenty-four hour broth cultures were streaked on Bordet-Gengou medium and incubated for twenty-four hours at 37°C. Smears were prepared from these plates and stained by the Neisser method long used for staining diphtheria bacilli. The Gram stain was also applied.

In all but one strain, granules were easily distinguished, the granules appearing as small, rounded, dark dots, with the remainder of the bacillus uniformly brown. Two or three granules were observed in individual cells, usually at or near the ends. In the longer rods as many as four or five were seen. Such stained films bore a strong resemblance to diphtheria slides. The preparations on first observation could easily be mistaken also for short streptococcus chains or grouped cocci.

The granule formation was best observed after 24 hours incubation. The intensity of granulation appeared to decrease after this period, with increased incubation. The approximate numbers of granular bacilli observed per microscopic field are shown in table 2.

TABLE 2

Showing the comparative prevalence of granules in the 12 strains examined

AGE OF CUL- TURE	NUMBER OF STRAIN											
	1	2	3	4	5	6	7	8	9	10	11	12
hours												
24	−	++++	+++	++++	++++	+++	++++	++++	+++	++++	++++	++++
48	−	+	+	++	+++	+++	++	++	+	+	+++	+++
72	−	−	+	++	+	+	+	+	+	+	+	+
96	−	−	−	−	−	+	−	−	+	−	−	−

++++ = granules in every cell.   − = none.

The granules were not demonstrable when the organisms were grown on standard Loeffler serum medium. Negative results were also obtained on blood agar, chocolate agar and liquid media.

CULTURAL CHARACTERISTICS

Plain broth. Growth was at best but meager; and many strains did not show any evidence of multiplication.

Carbohydrate broth. Group I. A medium to heavy turbidity, with or without sediment, developed after twenty-four hours of incubation at 37°C. The turbidity remained fairly constant even after a few days incubation, and was not readily thrown down completely at high speed centrifugation.

Group II. The turbidity in sugar broth was, as a rule, slight to moderate, and was accompanied with granular or viscous sediment at the bottom of the tube. Clearing of the medium occurred after twenty-four hours incubation. The deposited material often stuck to the side of the tube. Complete clarification was easily brought about by centrifugation.

Group III. The medium remained clear, as a rule, and there was a light cotton-like sediment at the bottom of the tube.

Litmus milk. Group I. The milk was usually coagulated after from twenty-four to forty-eight hours at 37°C. The curd was soft and smooth, with or without separation of whey.

Group II. Milk was as a rule coagulated much more slowly than by Group I.

Group III. Coagulation was very irregular. Some freshly isolated strains were slow to coagulate milk, but on continued artificial cultivation the curdling was a more or less constant feature. On the other hand, the coagulating property was often lost under these conditions. The litmus of litmus milk was usually decolorized during the coagulation process. In most instances the coagulation began at the bottom of the tube and progressed upward, while in some few cases the coagulation was general throughout the tube. Gas formation and peptonization of the casein were not brought about by any of the strains studied.

Potato medium. Growth was at best scant, being whitish and film-like along the needle track. Some strains produced a more pronounced growth on glycerol potato. Many strains did not grow at all on potato.

Gelatin. In some instances, faint, filiform or beaded growth along the needle track was seen after several days incubation at 20° to 22°C. At times there appeared to be a tendency to hair-like lateral branching. No liquefaction of gelatin was apparent. Many strains revealed no evidence of growth in gelatin.

Glucose agar slope. Growth occurred in the form of a thin whitish or gray film, or as small delicate, dew-drop colonies resembling those of the pyogenic streptococci.

Glucose agar plate. Growth was as a rule very delicate after twenty-four hours incubation at 37°C., and usually did not

acquire definite character until after incubation for another twenty-four hours. Several types of colony were observed.

Group I. The surface colonies were small, creamy or grayish-white, opaque, more or less glistening, butyrous, slightly convex, and oval to round, with entire border. Under the microscope they appeared yellowish-brown, structureless to finely granular, at times slightly streaked, round or almost round, with definite serrate margin. Deep colonies were opaque, brownish, comparatively large, and spindle to disc-shaped.

Group II. Surface colonies were grayish, dull, translucent, slightly raised, round, and somewhat smaller than those of group I, and of a firmer consistency. Under the microscope they were colorless, and homogeneous or slightly granular. Deep colonies resembled those of type I except that they were somewhat smaller.

Group III. Surface colonies were small, grayish, dull, translucent and flat, with rather rough surface and irregular outline. They bore some resemblance to the rough type of streptococcus colony. Microscopically, they were blackish, hair-like in texture and possessed an irregular border. The deep colonies were opaque blackish, small, and disc-like to spindle-shaped.

Group IV. Surface colonies were small, grayish, dull, translucent, slightly papillated, rather rough in texture, and round to irregular in outline. Under the microscope they resembled anthrax or young tetanus bacillus colonies. Deep colonies were very irregular and "fuzzy," long interlacing and hair-like threads radiating outward from the center. Often, small, brownish, disc-like or spindle-shaped colonies were also seen.

VARIATION IN COLONY FORMATION

L. acidophilus has been described in the literature as producing two distinct colony forms, the X and Y, or I and II types. Type I or Y is characterized by its definite outline, and its smooth and solid appearance under the microscope, while type II or X is a rough form, being finely filamentous, or "fuzzy," the filaments radiating out more or less from the more central portion of the colony. The two types are to a certain extent interchangeable, and modifications occur between the two extreme types.

In the present study these two colony types were observed in certain strains, particularly of Group IV. An intermediate type was also met with frequently, a so-called "X-Y" type. Crowded plates of the author's group I and II strains often exhibited both slightly fuzzy and definitely smooth colonies, while on plates having few colonies typical "X" or "Y" colonies which were so commonly observed in Group IV, were not formed.

Surface colonies on streaked agar plates also showed distinct differences, analogous to those of the poured plates, but not of the same definite mass morphology. Rather extensive experience with the agar plate pouring and streaking methods have convinced the writer that high dilution plates should be employed, and that stroke cultures on plates should be made along with the others.

INDOL FORMATION

No evidence of indol formation in tryptophan broth was observed.

RELATION TO TEMPERATURE AND ATMOSPHERIC OXYGEN

The optimum temperature for growth is about 37.5°C. All of the organisms are facultative anaerobes, being cultivated readily in the presence of ordinary atmospheric oxygen, as well as under the usual anaerobic conditions.

THERMAL DEATH POINT

Four different strains were employed in the twenty-four-hour broth cultures, heating being done at 50, 55, 60, 65, 70, and 75°C. for ten, thirty and sixty minutes. The organisms were killed at 65°C. in thirty minutes.

PATHOGENICITY

Five cubic centimeters of washed twenty-four-hour broth culture of the same 4 strains were injected subcutaneously into young guinea pigs. No definite pathological symptoms were induced by three of the four strains. Guinea pigs inoculated with the fourth strain died, however, from three to seven weeks after

the injections. The exact nature of the pathogenicity was not determined.

ACID FORMATION

One of the outstanding characteristics of aciduric organisms is their ability to produce relatively strong acid from fermentable substances and to tolerate high acidity, which few other organisms are capable of doing.

TABLE 3

Giving the H-ion concentrations of glucose yeast broth cultures after forty-eight hours incubation at 37°C.

	FINAL pH								
	3.3	3.4	3 5	3 6	3.7	3.8	4 0	4 1	4 4
Number of strains	2	23	7	27	1	24	2	1	1

TABLE 4

Showing the influence of initial H-ion concentration on growth and acid production

INITIAL pH	5 HOURS	10 HOURS	22 HOURS	48 HOURS	FINAL pH, 168 HOURS
3.5	−	−	−	−	3 6
4 4	−	±	++	+++	3 4
5.0	−	+	+++	++++	3.3
6 0	−	+	+++	++++	3 4
6 8	−	+	+++	++++	3.4
7.6	−	−	+	++	3 4
8 2	−	−	−	+	3 6

++++ = very good, +++ = good, ++ = moderate growth,+ = slight, ± = very slight, and − = negative growth.

During the course of these investigations it was found that determinations of acid production in sugar broths by over 100 strains of these organisms showed a final pH of from 4.8 to 3.0, most of the cultures registering between pH 4.0 and 3.4. One per-cent glucose yeast broth, pH 6.8, in 10-cc. amounts, was inoculated with 2 drops of material prepared from twenty-four-hour agar cultures, and incubated at 37°C. for forty-eight hours. The results with 88 strains studied are summarized in table 3.

All of the organisms were capable of producing relatively strong acidity within forty-eight hours, varying within a range of pH 3.3 and 4.4.

In another experiment the influence of H-ion concentration of the medium on the growth and acid production of the organisms was determined with 4 strains. Tubes containing 10 cc. of 2 per cent glucose yeast broth having different ranges of pH (8.2 to 3.5) were inoculated with a drop of the inoculum prepared from twenty-four-hour agar cultures, and the tubes incubated at 37°C. Growth was measured by the turbidity produced in the tubes. The results are shown in table 4.

The best growth occurred in the acid range between pH 6.8 and 5.0; good development also took place at pH 4.4. The growths were markedly inhibited by an alkaline reaction, and on the other hand, no growth occurred at pH 3.5. This evidence would indicate that media having a pH of 3.5, which McIntosh and his co-workers employed for isolation of the aciduric organisms, are too acid to insure growth. The final H-ion concentrations after 168 hours incubation were apparently essentially the same in the different tubes. It would seem from the writer's observations that the optimum reaction of media for routine cultivation of the organisms is about pH 6.8.

FERMENTATION REACTION

Rahe (1918) classified *B. acidophilus* Moro according to ability or inability to ferment glucose, maltose, lactose, sucrose and raffinose. The organisms quite generally fermented sucrose and raffinose. On the other hand, McIntosh and his co-workers (1922, 1924) reported that *B. acidophilus-odontolyticus* does not readily attack raffinose and dextrin, and sucrose to a limited extent only. Clarke (1924) found that his organism, *Strept. mutans*, fermented raffinose, mannitol, salicin and inulin. Bunting and Palmerlee (1925) classified acid-forming organisms of dental and salivary origin according to the Rahe system. They labelled their strains *B. acidophilus*. They found, however, that raffinose was fermented by only one out of 25 strains.

Dunham peptone solution was used by the writer as a basic

medium. To this the different test substances were added in 1 per cent concentration together with Andrade's indicator, and the medium distributed in 3 cc. quantities in small test tubes. Sterility was determined by incubating the sterilized tubes at 37°C. for two days. Two drops of the inoculating material were introduced into each tube, and the tubes incubated, together with uninoculated tubes serving as control, at 37°C. for seven days. The purity of the cultures was determined by plating portions of the inocula. The tests were repeated when a negative reaction was obtained. In this experiment 52 strains were used, with the following results.

1. Raffinose fermenters as a rule fermented sucrose.

TABLE 5

Showing the reaction on different carbohydrates, etc., of the various strains of the present collection

	DEXTROSE	LEVULOSE	GALACTOSE	RHAMNOSE	MALTOSE	LACTOSE	SUCROSE	RAFFINOSE	DEXTRIN	STARCH	SALICIN	MANNITOL	DULSITOL	SORBITOL	MILK (LITMUS)*
Positive	52	52	51	38	50	47	35	26	5	0	27	28	3	29	29
Negative	0	0	1	14	2	5	17	26	47	52	25	24	49	23	23

* Coagulation.

2. Mannitol fermenters always attacked salicin and sorbitol, but did not ferment raffinose readily.

3. Dextrin was usually fermented when salicin was.

4. Dextrin-salicin fermenters which did not attack either mannitol or sorbitol often fermented raffinose.

5. Only two strains failed to break down maltose, and only one did not ferment galactose.

6. Milk was usually coagulated by mannitol-sorbitol-salicin fermenters.

7. Gas formation was not observed in all strains studied.

As regards a possible relationship between the fermentation reactions and the morphological and cultural findings, it may be

said that there were certain general correlations when certain sugars were used, and when certain strains were employed.

Raffinose and sucrose fermentation appeared to be of no real significance in distinguishing these organisms from known strains of *L. acidophilus* Moro without further study, owing largely to the existence of so many different fermentation groups in the present study. (See table 5.)

It would appear, therefore, that this group of organisms cannot be classified according to Rahe's method for aciduric organisms. Indeed, there is much doubt as to the value of the carbohydrates employed by Rahe in the present identification problem.

<div align="center">AGGLUTINATION REACTIONS</div>

Agglutination experiments will be discussed in another paper. It should be pointed out here, however, that the aciduric organisms associated with dental caries, and those which were isolated from saliva, have been found to admit of definite grouping on the agglutinability basis.

No reference has thus far been made to the taxonomic relationship of these organisms to *L. acidophilus* of intestinal origin. It may be of interest at this point to state that the group studied here is markedly different in certain important respects from *L. acidophilus*. This phase will be presented in a future report.

<div align="center">GENERAL DISCUSSION AND CONCLUSIONS</div>

High-acid-tolerating organisms were found to be of almost constant occurrence in tooth enamel which was undergoing an early or initial active pathological process. They were also generally present in the saliva of persons harboring carious teeth. On the other hand, they were found to be relatively rare in the saliva of non-carious mouths.

The relatively high acidity produced by some of these aciduric forms is sufficient to cause decalcification of the enamel in *in vitro* experiments, as will be shown later. However, no one has as yet apparently succeeded in producing dental caries with these organisms in *in vivo* experiments. In spite of their common occurrence in active pathological parts of the teeth and in saliva,

it is impossible as yet to offer final proof that these organisms are directly responsible for the production of tooth decay. On the other hand, failure to produce such a pathological condition *in vivo* is no proof that they are not associated in an intimate way with the disease.

The establishing of a definite species of bacterium and its exact relation to caries constitutes a complex problem. Observations on the morphological types presented by McIntosh and his co-workers (1922, 1924), and by Rodriguez (1922), and on the morphological species described by Howe and Hatch (1917), do not take into account the important question of variation, with which the writer and others have for some time been engaged.

On the other hand, Bunting and Palmerlee (1925) recognized considerable morphological variation, which they regarded as phases of "pleomorphism." They have placed all acid-forming organisms derived from teeth and saliva in a single species, "*B. acidophilus*," and classified them according to Rahe's fermentation method. Such a classification would appear to the writer decidedly premature, and may lead to still more confusion in this already chaotic field.

It is impossible to say definitely whether the different organisms described by various workers are entirely distinct species, or whether they are different forms of one or the same species.

The organisms isolated from carious teeth and from saliva, and studied here, were not "highly pleomorphic," though they exhibited some variation. Several different morphological, cultural, biological and serological groups have been established, which would naturally suggest that there are several different species or types among these strains. This subject will be discussed in greater detail in a subsequent paper.

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THE FERMENTOMETER

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In 1906, Slator described a little instrument to determine the rate of yeast fermentation in short intervals. He measured the pressure of CO_2 produced in a closed container by means of a mercury manometer. The method was very simple and gave the possibility of determining the rate of fermentation in five to ten minutes. The only essential deviation from Slator's method in the "Fermentometer" described here is that Slator evacuated the apparatus and measured the decrease of vaccuum while in the following experiments, no vacuum was applied and the pressure measured was surplus pressure caused by the carbon dioxide produced by the yeast.

The apparatus consists in a flask or bottle closed with a perforated rubber stopper (ground glass would be better) with a glass tube which is connected by rubber tubing with the manom-. eter. This is a simple glass tubing manometer, open at both ends, with a glass stopcock to release the pressure. In order to be able to speak of this instrument in one word, we have called it fermentometer, since Slator did not give it any name.

This little instrument has proved very convenient for the study of the influence of various factors on the rate of fermentation. Since in most cases, the total observation times are short, not more than a few hours, and since large quantities of yeast will always be used to get rapid development of pressure and to prevent multiplication, it is permissible for most experiments to use the ordinary yeast cake which is not a pure culture, but sufficiently pure as far as gas formation is concerned.

The advantage of this instrument is the possibility of determin-
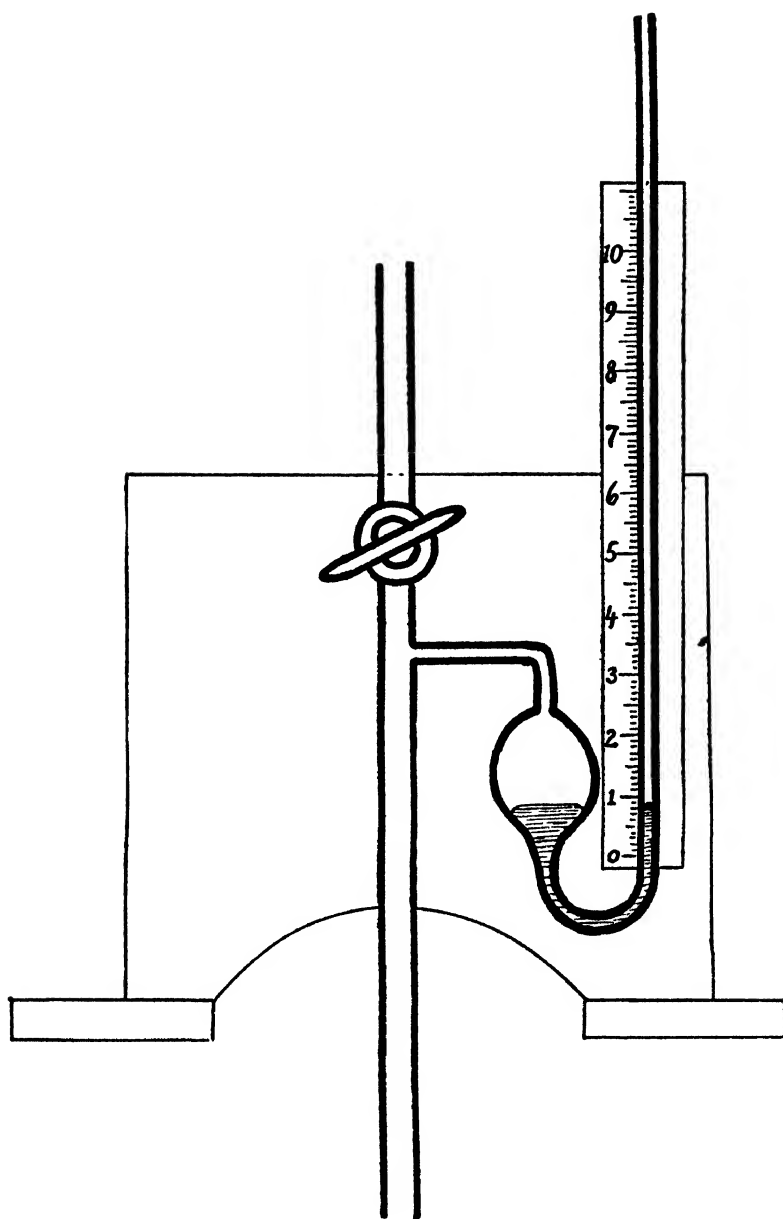
FIG. 1. THE FERMENTOMETER

ing rates of fermentation in a very short time, much shorter than with any other fermentation, and entirely independent of multiplication. In most fermentations, the products formed in a given time interval are produced by an increasing number of cells, and it takes a complicated formula to compute the fermenting capacity of the single cell as a measure of the rate of fermentation independent of multiplication (Rahn, 1911; Buchanan and Fulmer, 1918). Besides, neither of these two formulas is really correct, because they are based on the assumption that bacteria multiply in a strictly exponential way, in the order $a \cdot 2^x$; while this is correct for the first part of growth in a new culture, it is doubtless not correct for that part of the curve where most of the fermentation is observed, i.e., that part when the growth curve has reached its point of inflexion and the rate of growth is decreasing.

TECHNIQUE

In order to get a measurable increase in pressure, it seems necessary to have as small an air space in the apparatus as possible. The author preferred small Erlenmeyer flasks holding when filled to the rim about 100 cc. of water. The connecting rubber tubing should not be longer than necessary to allow vigorous shaking. This shaking is necessary to drive the excess carbon dioxide out of the supersaturated solution. The author followed Slator's method by putting one teaspoonful of glass beads into each flask. By using the 100 cc. flask with glass beads and 50 cc. of sugar solution to which 5 cc. of a suspension of 1 yeast cake (about 12 grams) in 50 cc. of water, i.e., 1 to 1.5 grams of yeast per flask was added, the author obtained pressures of 10 to 25 mm. in five minutes, depending upon temperature and other conditions. This is about the right pressure for measurements.

After trying various ways, it finally was decided to leave the yeast culture undisturbed for four and one-half minutes, shake the culture for half a minute vigorously avoiding however, as far as possible, entrance of the liquid into the glass and rubber tubing, read the manometer, and wait for another four and one-half minutes to repeat the procedure. This makes it possible to observe 5 fermentometers in one experiment simultaneously.

TABLE 1

Parallel experiments, 1 gram yeast in 55 cc. glucose solution

TIME AFTER ADDING YEAST TO SUGAR SOLUTION		MERCURY PRESSURE IN 5 MINUTES					AVERAGES				
		A	B	C	D	E	A	B	C	D	E
hours	minutes	mm.	mm.	mm.	mm.	mm.					
	0–5	15.5	17	16.5	12	8					
	5–10	20.5	15	14.5	18	7					
	10–15	18	15.5	15.5	18	7					
	15–20	17.5	15.5	15.5	17.5	New	Pressure released after every reading				
	20–25	17	15	13.5	17	rubber stopper					
	40–45	18	15	Lost	16	14					
	45–50	17.5	15.5	13	18.5	15					
	50–55	18	15	14	18.5	17.5					
	55–60	13.5	12.5	13	13.5	12.5	13.5	13.0	13.0	13.25	12.25
	60–65	13.5	13.5	13	13	12					
2	0–5	21	15	15.5	20	15					
	5–10	21.5	18	15.5	20	17	Pressure released after every reading				
	10–15	21.5	19	17.5	21	19					
	15–20	22.5	17	17.5	20.5	19					
	20–25	13	13.5	14	15.5	12					
	25–30	15.5	15.5	14.5	13.5	13					
	30–35	14	13	14.5	15.5	13.5	14.6	14 2	14:4	14.9	13.2
	35–40	15	15	14	15	13.5					
	40–45	14.5	14	15	15	14					
	45–50	15.5	Broken	14	15	13					
5	0–5	18		21	19	20					
	5–10	24.5		23	24	23	Pressure released after every reading				
	10–15	24.5		21.5	24	22					
	15–20	13.5		13.5	16	13.5					
	20–25	14.5		15	14	14.5					
	25–30	15		14	14	15	14.9		14.4	15.0	14.3
	30–35	15		15	15	13.5					
	35–40	15		14	16	14.5					
	40–45	16		15	15	15					
7	30–35	22.5		18	25	19.5	Pressure released after every reading				
	35–40	25		20.5	24	20.5					
	40–45	12.5		12.5	14	12.5					
	45–50	12.5		12.5	13.3	13.2					
	50–55	13		12.5	13.3	13.2					
	55–60	12.5		12.5	13.3	13.2	13.1		12.9	13.3	13.2
	60–65	13.5		14.5	13.3	13.2					
	65–70	14.5		12	13.3	13.2					
	70–75	13.5		13.5	13.3	13.2					

The frequency of shaking and reading and of releasing the pressure will of course depend upon the rate of fermentation.

Table 1 gives a typical experiment showing a comparative test of 5 fermentometers. All flasks were treated exactly alike containing each 50 cc. of an 8 per cent glucose solution and 5 cc. of yeast suspension (10 grams yeast cake in 50 cc. of water), so that each flask received about 1 gram of yeast. All cultures stood in the 30° incubator room in water baths of 27.5°C.

The first five minutes cannot be counted as full because some of the CO_2 produced is dissolved in the medium, and only after this is saturated, can the actual measurement begin. It takes less than five minutes to develop sufficient CO_2 to saturate the medium. The first measurements show no very good agreement, even if we omit the last observation which was spoiled by a fine scratch in the rubber stopper. In the beginning, pressure was released after every reading, then, after fifty minutes, pressure was not released, with the result that the data became at once fairly constant.

The yeast was then left to itself for an hour, with the glass stopcock of the manometer open. Two hours after the first mixing of yeast and sugar solution, the pressure was measured again, with the same result that by letting the pressure accumulate, the results became more stable. The same was true after five hours. The reason is probably that with pressure at zero, the manometer does not indicate the pressure accurately while as soon as pressure is developed, the readings are reliable.

Another bothersome feature has not been fully accounted for as yet, that is the gradual increase in the rate of fermentation as evidenced by the average results of the first experiment. It does not seem to be caused by the pH adjustment because this slow increase has been observed at all pH values tried. The average of all 5 experiments is 10 per cent higher at two hours than at one hour, and 11.7 per cent higher after five hours, while after seven hours, the rate of fermentation decreases on account of the accumulation of the alcohol. This increase in the rate of fermentation may be due to a rejuvenation process in the old yeast, though no nitrogenous material was given nor could any dead cells be detected by the methylene blue test which might be the source of nitrogen.

For most experiments, it is sufficient to compare the pressure readings directly, provided that the flasks are of uniform size. Occasionally, however, the absolute quantities of CO_2 produced during the experiment may be wanted. This can be ascertained by measuring the total volume of the enclosed gas in the apparatus, i.e., in flask, rubber tubing and fermentometer. By filling the flask to the rim with water, and then placing the rubber stopper and glass tube in their usual position, and by filling also rubber tubing and manometer with water to the same amount which is ordinarily filled with gas, the water volume gives the total en-

TABLE 2

Corrected volumes of CO_2 produced by yeast

	A	B	C	D	E
Total volume of flask, stoppered, + glass tube, cc.	103	103	103	99	104
Gas volume of manometer	7.5	3.5	6.5	7	10
Gas volume of rubber tubing	2.5	2.5	2.5	2.5	2.5
Volume of culture liquid	55	55	55	55	55
Volume of glass beads	2	2	2	2	2
Total enclosed airspace	56	52	55	51.5	59.5
Real volume of CO_2 produced per 5 minutes — After 1 hour	0.995	0.89	0.94	0.90	0.96
After 2 hours	1.08	0.97	1.04	1.01	1.03
After 5 hours	1.10		1.04	1.02	1.12
After 7.5 hours	0.965		0.93	0.90	1.03

closed volume. From this, we have to deduct the volume of the solution used in the experiment, including the glass beads. The remaining volume is the enclosed gas space over the culture, which we shall call v. By the formation of x cc. of CO_2, the pressure increases p mm. Since pressure times volume is constant, we must have

$$(v + x)\ 760 = v\ (760 + p)$$

This equation means that the original gas volume, increased by x cc. of CO_2 at normal pressure, will be reduced to the original gas volume v if the pressure is increased by p mm.

The total CO_2 formed is therefore $x = \dfrac{v.p.}{760}$ cc.

Applying this to the above experiment, we find the results given in table 2.

This means that each flask containing about 1 gram of yeast produces about 1 cc. of CO_2 in five minutes which corresponds (uncorrected for temperature and moisture) to 3.95 mgm. This means 47.4 mgm. of CO_2 per gram yeast per hour, and about the same amount of alcohol. This quantity in 55 cc. corresponds to 0.086 per cent per hour.

This little instrument has been used by Slator to measure the influence of temperature, of sugar concentration and of stimulants upon the rate of alcoholic fermentation. The author has used it to study the influence of temperatures above the optimum, and the influence of alcohol upon the rate of fermentation; he expects to use it for the study of the theory of chemical stimulation.

The fermentometer will prove a useful, time-saving and inexpensive little instrument wherever the rate of alcoholic fermentation is being studied from a physiological viewpoint.

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THE DECREASING RATE OF FERMENTATION

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It is a general experience that fermentations come to end, even before all the fermentable substrate is used up. There are various explanations for this gradual retardation and ultimate cessation of the rate of fermentation; it may be due to lack of substrate, or to toxic action of fermentation products, or to some other toxic cell product not generally considered a fermentation product, or to some biological change of the cells. While all these reasons might occasionally occur, the common cause of the decrease in the rate of fermentation ordinarily is either the accumulation of fermentation products, or the decrease of fermentable substrate. The concentration of the substrate must become very low before it exerts a retarding influence because the rate of fermentation is independent of the concentration of the substrate.

The independent relation of alcoholic fermentation to sugar concentration was demonstrated by Slator as early as 1906. His data show that the rate is nearly constant if the sugar concentration exceeds 1.5 per cent. As far as the author has been able to find, this rule has not been proven for any other type of fermentation.

The lack of dependence of the fermentation upon the concentration is of considerable significance for the understanding of fermentation and growth. It is, therefore, desirable to have more proof for this rule. Some very good examples can be found in Rubner's studies (1912) on the alcoholic fermentation. A good deal of interesting material has been gathered in this treatise which fills a whole volume. But it is hidden in a queer phraseology, and that may account for the fact that these data are hardly known in American literature.

Rubner's object was primarily the study of the energy relations in the alcoholic fermentation, and later, he used the caloric method, once developed, for other studies where a chemical analysis would have proved more useful. He put a definite amount of yeast, usually 5 grams, in 250 cc. of sucrose solution and studied the process of the fermentation by the increase of temperature in the culture which was placed in a thermos bottle. The thermometer readings were computed into calories, and all data on fermentation in this volume are given in calories. Since it is known that 1 gram of sucrose yields 149.5 calories in the alcoholic fermentation (average of all of

TABLE 1

Table of corrections

	CALORIES MEASURED IN CULTURE + TOLUOL BY RUBNER					CALORIES SUBTRACTED FOR CORRECTION AS DUE TO INVERTASE				
	Sugar concentration									
	20 per cent	10 per cent	5 per cent	2 5 per cent	1.25 per cent	20 per cent	10 per cent	5 per cent	2 5 per cent	1.25 per cent
hours							*f*			
0–2	425	320	196	88	Not meas- ured	399	200	100	50	25
2–4	84	23	27	2		57	28	14	7	4
4–6	24	6	0	0		8	5	2	1	0
6–8	9	7	0	0		1	0	0	0	0
8–10	1	0	0	0		0	0	0	0	0
Total heat produced	543	356	223	100		465	233	116	57	29
Invertase heat............	465	233	116	57						
Zymase heat............	78	123	107	43						

Rubner's data) and 1 gram of glucose yields 140.2 calories, it would be easy to change calories into per cents of sugar, if complications did not interfere. Unfortunately, Rubner used sucrose in all his experiments, and sucrose is readily inverted by the invertase of the yeast. The inversion of sucrose is exothermic, and yields 9.3 calories per gram. The inversion is independent of the fermentation, and proceeds much faster, and this means that during the first hours of fermentation, the heat produced comes partly from alcoholic fermentation and partly from sugar inversion. This last amount can be estimated, however. It so happens that Rubner was anxious to make a distinction between the alcoholic fermentation caused by the free enzyme "zymase" and the

fermentation brought about by the protoplasm itself. He, therefore, made parallel experiments using the same amounts of yeast and sugar solution with addition of toluol. The heat produced in the presence of toluol was largely due to invertase but partly to zymase. These experiments give a good conception of the rate of invertase action, and

TABLE 2

Alcoholic fermentation of sugar solutions of different concentration by 5 grams of yeast

	TOTAL CALORIES IN 250 CC. CORRECTED FOR INVERTASE					PER CENT SUGAR FERMENTED					FERMENTATION CONSTANT $k = \frac{1}{t} \log \frac{2L}{2L - x}$			
	Sugar concentration													
	20 per cent	10 per cent	5 per cent	2 5 per cent	1 25 per cent	20 per cent	10 per cent	5 per cent	2 5 per cent	1 25 per cent	20 per cent	10 per cent	2 5 per cent	1.25 per cent
hrs														
2	657	591	643	526	344	1.88	1.69	1.83	1.50	0.98	0.0207	0.0187	0.0203	0.0165
4	1,168	1,164	1,154	691	411	3.33	3.32	3.29	1 97	1.17	0.0193	0.0192	0.0181	
6	1,621	1,624	1,464	757	434	4.63	4.64	4 18	2.16	1.24	0.0187	0.0187		
8	2,004	2,071	1,562	780	448	5.72	5.90	4.46	2.23	1.28	0.0179	0.0185		
10	2,340	2,459	1,597	808	455	6.68	7.01	4.55	2.31	1.30	0.0172	0.0183		
12	2,652	2,711	1,649	845	465	7.57	7.73	4.70	2.41	1.33	0.0168	0.0172		
14	2,923	2,921	1,681	869	473	8.34	8.34	4.80	2.48	1.35	0.0163	0.0163		
16	3,170	3,079	1,688	891	480	9 05	8 78	4.81	2.54	1.37	0.0160			
18	3,377	3,193	1,704	912	486	9.63	9.11	4.86	2.60	1.38	0.0154			
20	3,597	3,263	1,714	922	489	10.27	9.32	4.89	2.63	1.39	0.0152			
22	3,815	3,312	1,725	925		10.88	9.45	4.92	2.64		0.0151			
24	4,000	3,335	1,729			11.42	9.52	4.93			0.0144			
30	4,522	3,345				12.90	9.55				0.0119			
40	5,249					14.98					0.0144			
50	5,804					16.56					0.0145			
60	6,205					17.70					0.0147			
70	6,426					18.34					0.0142			
80	6,559					18.71	Average				0.0160	0.0181	0.0192	0.0165
90	6,584					18.80								

make it possible to work out tables of correction. The *total* heat produced from inversion is known, and the uncertainty rests only in the correct distribution of this amount over the various time intervals of the experiment. To give a conception of how this was done, the following table 1 shows the heat produced by yeast + sugar + toluol, and the heat used for the correction for invertase in Rubner's data by the

author. From the data given by Rubner on page 105, the values of table 1 are subtracted; thus, we obtain the left section of table 2. The data are then divided by 3505 to change calories to per cents of sugar; the central part of the table shows the total amounts of sugar fermented at the times given. In this way, we obtain very useful tables.

The central part of table 2 shows the amount of sugar in 5 different dilutions decomposed by the same amount of yeast (5 grams in 250 cc. of sugar solution). The sugar concentration varied greatly, but the amounts of sugar decomposed are quite uniform in the different solutions until the concentration falls below 1.5 per cent. This event is indicated in the table by a cross line. It occurs with the 2.5 per cent solution after two hours, with the 5 per cent solution after four hours, with the 10 per cent solution after fourteen hours. After four hours, the sugar quantity decomposed by the yeast is the same in the 20, 10 and 5 per cent solution. The two highest concentrations are as nearly parallel in their fermentation as can be expected up to the 14th hour. This is a very good illustration of the independence of the fermentation from the sugar concentration.

But the rate does not stay uniform during the entire experiment. During the first four hours, 3.33 per cent of sugar are decomposed, during the next four hours 2.39 per cent, and in the following four hours only 1.85 per cent. The rate of fermentation decreases. This may be due to the alcohol formed, because it cannot be caused by the decrease of sugar, for we have just seen that the sugar concentration does not affect the rate of fermentation.

It seems desirable to bring this retarding effect of the alcohol into a definite formula. The most simple form would be the assumption that the rate is decreased proportional to the amount of alcohol, or that the rate is proportional to the original rate decreased by a certain amount which is proportional to the alcohol already present. This might be written in the following way:

Rate at any time = Initial Rate (1 − k × alcohol concentration)

The question would be how to express the alcohol concentration. Since a fermentation comes to a stop at a fairly definite con-

centration, which is characteristic for the organism used, the alcohol concentration can be best expressed in terms of this limiting concentration which we will designate as L.

$$\text{Rate at any time} = \text{Initial Rate}\left(1 - \frac{\text{alcohol}}{L}\right)$$

If the alcohol concentration has reached the limiting concentration, the fraction $\frac{\text{alcohol}}{L}$ equals 1, and the rate of fermentation equals Initial Rate $\times$ (1 − 1) which is zero; the fermentation comes to a stop.

We can go one step further and write the rate of fermentation in its mathematical form:

$$-\frac{dx}{dt} = k \cdot Y\left(1 - \frac{\text{alcohol}}{L}\right)$$

which means that the rate with which the sugar concentration is changed, is proportional to the amount of yeast Y, and is independent of the sugar concentration which therefore does not enter the equation at all, and is retarded by alcohol in the manner discussed above. The alcohol must be expressed in terms of sugar. Since one gram of sugar gives 0.511 gram of alcohol and 0.489 gram of CO_2, we can with sufficient accuracy substitute

$$\text{alcohol} = \frac{x}{2}$$

because x was the amount of sugar decomposed. Thus we get the equation

$$-\frac{dx}{dt} = k \cdot Y\left(1 - \frac{x}{2L}\right)$$

$$= \frac{k}{2L} Y(2L - x)$$

Y, the amount of yeast, is constant in all these experiments of Rubner's because more yeast was ordinarily added than would normally grow in such a solution, and besides, there is no nitrog-

enous material for yeast to feed on. Therefore, the entire expression $\dfrac{k}{2\,L} \cdot Y$ is constant, and may be substituted by another constant K. The equation

$$-\frac{dx}{dt} = K(2L - x)$$

gives upon integration

$$Kt = -\ln (2L - x) + C$$

for $t = 0$, $x = 0$, and thus we obtain

$$0 = -\ln 2L + C$$

Subtracting this equation from the above, we obtain the final equation

$$Kt = \ln \frac{2L}{2L - x}$$

The accuracy of this equation can be tested by computing the $K-$ values for all the above data. It is necessary, however, to know the value of L. This can be obtained approximately from Rubner's statement that in the above concentrations of sugar, all sugar had disappeared in the end, but it took the 20 per cent solution eighty-eight hours to ferment the last of it. This indicates that the yeast could not have fermented much more than 20 per cent sugar. Twenty per cent of sugar would yield the theoretical value of 10.2 per cent alcohol. This value has been used to compute the fermentation constants in the right section of table 2.

These fermentation constants express the rate of reaction at a definite time, corrected for the retarding effect of alcohol, and they should be constant during the entire course of the fermentation until the sugar concentration falls below 1.5 per cent. This is not quite the case. The computed data show a slow but distinct decrease. Probably this is not due to the inaccuracy of the data on fermentation, though they are subject to considerable error through the method of measurement as well as

through the method of correction. But the fermentation constants are calculated with the assumption that the yeast cells and their zymase content remain unchanged, and this is not entirely correct as will be seen in table 5.

Another one of Rubner's experiments deserves especial interest, namely, the one where he tested the inhibiting factor of alcohol (Rubner's, page 88) by measuring the heat of fermentation

TABLE 3

Fermentation with the addition of alcohol

	250 CC. OF CULTURE GAVE						PER CENT OF SUGAR FERMENTED AS COMPUTED FROM TOTAL CALORIES		K	
	Calories for each 2 hours		Calories for each 2 hours, corrected for invertase		Total calories corrected					
	Alcohol added									
	4 72 per cent	0	4.72 per cent	0	4 72 per cent	0	4 72 per cent	0	4 72 per cent	0
hours										
2	435	637	235	437	235	437	0.67	1.25	0.0137	0.0096
4	224	290	196	262	431	699	1.23	1.99	0.0103	0.0111
6	161	287	155	282	586	981	1.67	2.81	0.0120	0.0107
8	140	254	140	254	726	1,235	2.18	3.53	0.0120	0.0103
10	141	242	141	242	867	1,477	2.47	4.22	0.0111	0.0101
12	140	213	140	213	1,007	1,690	2.86	4.83	0.0109	0.0098
Average...									0.0117	0.0103

in a culture to which a known amount of alcohol had been added. The rate of fermentation in this case would be

$$\frac{dx}{dt} = \frac{k \cdot Y}{L}\left(L - \frac{x}{2} - a\right)$$

where a is the concentration of the alcohol added.

$$\frac{dx}{dt} = \frac{k \cdot Y}{2L}(2L - 2a - x)$$

$$Kt = \ln\frac{2L - 2a}{2L - 2a - x}$$

This formula should apply to the following data representing the fermentation of a 10 per cent sucrose solution by 5 grams

TABLE 4

Fermentation by different quantities of yeast

	PERCENTAGE OF SUGAR FERMENTED							
	20 per cent sucrose				10 per cent sucrose			20 per cent
	8 grams yeast	4 grams yeast	2 grams yeast	1 gram yeast	10 grams yeast	5 grams yeast	1 gram yeast	1 gram yeast
After 2 hours	1.60	0.80	0.25	0.03	1.61	1.09	0.46	0.29
After 4 hours	2.96	1.64	0.76	0.28	3.32	1.85	0.76	0.56
After 6 hours	4.12	2.30	1.20	0.48	4.64	2.58	0.98	0.81
After 8 hours	5.13	2.96	1.61	0.74	5.92	3.26	1.18	1.02
After 10 hours	6.08	3.59	2.03	0.99	7.03	3.92	1.38	1.22
After 12 hours	7.00	4.18	2.46	1.19	7.75	4.54	1.56	1.41
After 14 hours	7.86	4.75	2.84	1.40	8.35	5.10	1.73	1.60
After 16 hours	8.67	5.22	3.21	1.60	8.80	5.64	1.80	1.80
After 18 hours	9.47	5.76	3.57	1.80	9.12	6.12	2.09	1.99
After 20 hours	10.18	6.20	3.92	1.97	9.32	6.58	2.24	2.14
After 22 hours	10.80	6.62	4.22	2.12	9.45	7.01	2.39	2.28
After 24 hours	11.45	7.01	4.53	2.29	9.57	7.42	2.54	2.42
	FERMENTATION CONSTANTS							
After 2 hours	0.0177	0.0087	0.0028	0.0002	0.0177	0.0118	0.0045	0.0030
After 4 hours	0.0170	0.0092	0.0039	0.0014	0.0193	0.0102	0.0041	0.0024
After 6 hours	0.0163	0.0087	0.0044	0.0016	0.0187	0.0104	0.0035	0.0029
After 8 hours	0.0157	0.0085	0.0044	0.0020	0.0187	0.0094	0.0032	0.0028
After 10 hours	0.0154	0.0084	0.0045	0.0021	0.0183	0.0092	0.0030	0.0026
After 12 hours	0.0163	0.0083	0.0046	0.0022	0.0183	0.0091	0.0029	0.0026
After 14 hours	0.0151	0.0082	0.0046	0.0022	0.0163	0.0089	0.0028	0.0025
After 16 hours	0.0150	0.0081	0.0046	0.0022	0.0154	0.0087	0.0027	0.0025
After 18 hours	0.0151	0.0080	0.0046	0.0022	0.0143	0.0086	0.0026	0.0024
After 20 hours	0.0150	0.0078	0.0045	0.0022	0.0132	0.0085	0.0025	0.0024
After 22 hours	0.0148	0.0077	0.0045	0.0021	0.0123	0.0083	0.0025	0.0023
After 24 hours	0.0149	0.0076	0.0045	0.0021	0.0114	0.0082	0.0024	0.0023
Averages........	0.0157	0.0083	0.0043	0.0021	0.0182	0.0093	0.0031	0.0025

of yeast, where 4.72 per cent of alcohol were added to one of the two experiments.

No special experiments were carried on by Rubner to determine the heat of inversion, but it seems quite safe to use the same data as in pre-

vious experiments for the correction. If this is done we get a fairly satisfactory constancy for the values of k, whether computed in the culture without or with the special addition of alcohol.

The $K-$ values of table 3 show the same slight decrease as was noticed in table 2. But they prove the general point that the equation is applicable to the data obtained. The $K-$ values of the experiment with the addition of alcohol are a little higher than in the normal fermentation. The most probable explanation for this is slight differences in the quality or quantity of yeast used in the two experiments. While differences in the assumption of L make very little impression upon the error, the yeast concentration is the deciding factor.

This is shown by the following two series of experiments with different amounts of yeast which are compiled here in table 4, while Rubner treats the two divisions as entirely separate. The fermentation constants show the gradual decrease with time only in the higher concentrations. In the lower yeast concentrations, there is an increase followed by a very slight decrease. The increase is probably caused by a wrong correction. The subtraction for the invertase may have been too large at first and too small in the later hours of fermentation, while the total correction is accurate.

The rates of reaction follow very closely the assumption that they are directly proportional to the yeast concentration. The only deviation is the 1-gram culture of the second series whose rate is almost twice as high as should be expected. An explanation cannot be given. The rate of the three experiments with 1 gram of yeast, one in 10 per cent sucrose and the other two in 20 per cent sucrose is fairly uniform, 0.0021, 0.0025 and 0.0031.

Another experiment of Rubner's will be of interest here; it was made to determine the gradual deterioration of the fermenting capacity of yeast in sugar solutions without nitrogenous food. Five grams of yeast were placed in 250 cc. of a 10 per cent sucrose solution as usual; after twenty-four hours, the yeast was separated from the medium by centrifugation, and was suspended in 250 cc. of fresh sucrose solution; this was repeated 5 times. This

treatment compelled the same yeast to ferment again and again without any chance of replacing the protoplasm decomposed by endogenous catabolism.   Parallel series were made to determine

TABLE 5

Fermentation by 5 grams of yeast at 36° to 38°C. in 250 cc. of sugar solution renewed every day for six days

	PER CENTS OF SUGAR FERMENTED					
	First day	Second day	Third day	Fourth day	Fifth day	Sixth day
After 2 hours	1.25	0.43	0.55	0.42	0.19	0.11
After 4 hours	2.19	0.90	0.73	0.47	0.17	0.12
After 6 hours	3.07	1.34	0.97	0.57	0.21	0.15
After 8 hours	3.78	1.71	1.15	0.63	0.28	0.19
After 10 hours	4.49	2.12	1.37	0.76	0.36	0.25
After 12 hours	5.06	2.48	1.55	0.84	0.43	0.31
After 14 hours	5.51	2.83	1.71	0.92	0.49	0.36
After 16 hours	5.90	3.15	1.86	0.98	0.54	0.40
After 18 hours	6.20	3.43	1.97	1.03	0.59	0.44
After 20 hours	6.46	3.71	2.07	1.08	0.64	0.48
After 22 hours	6.64	3.97	2.19	1.13	0.67	0.50
After 24 hours	6.70	4.16	2.30	1.19	0.74	0.52
	FERMENTATION CONSTANTS					
After 2 hours	0.0137	0.00452	0.00579	0.00452	0.00203	0.00117
After 4 hours	0.0123	0.00478	0.00395	0.00228	0.00075	0.00064
After 6 hours	0.0118	0.00489	0.00346	0.00205	0.00075	0.00053
After 8 hours	0.0111	0.00473	0.00316	0.00170	0.00075	0.00051
After 10 hours	0.0108	0.00473	0.00302	0.00165	0.00077	0.00054
After 12 hours	0.0103	0.00468	0.00285	0.00152	0.00077	0.00055
After 14 hours	0.0097	0.00463	0.00273	0.00143	0.00075	0.00055
After 16 hours	0.0093	0.00454	0.00259	0.00134	0.00073	0.00054
After 18 hours	0.0088	0.00444	0.00243	0.00125	0.00071	0.00053
After 20 hours	0.0082	0.00435	0.00229	0.00118	0.00069	0.00052
After 22 hours	0.0080	0.00426	0.00224	0.00113	0.00066	0.00048
After 24 hours	0.0072	0.00411	0.00216	0.00109	0.00066	0.00047
Average from 8-18 hours	0.01000	0.00463	0.00280	0.00148	0.00074	0.00054

alcohol formed, nitrogen lost from the yeast cells, and to make plate counts.

Table 5 gives the course of fermentation on each of the six days of the experiment.   It is quite evident that the yeast weakens.

The sugar decomposed during the sixth day is less than one-tenth of that decomposed during the first day.

The fermentation constants are also quite interesting. On the second day, the yeast starts with a rate of 0.0045 while before centrifugation, the rate was 0.0072. This is the rate already corrected for the inhibition through alcohol. It is very good evidence for the assumption made above that the slow decrease of the fermentation constants is brought about by a gradual decrease of the yeast activity or their zymase content. On all later days, the rate of fermentation during the first two hours is exceptionally high, and a good deal higher than it was at the twenty-fourth hour of the preceding day, just before centrifugation. This is not caused by alcohol, because its influence has already been eliminated from the fermentation constant. It is not very likely to be due to traces of nitrogenous food because such influence would last longer than two hours. It may be due to faulty correction; since it was impossible to estimate the amount of invertase left in the yeast after several repeated leachings in the nitrogen-free sugar solutions, the total amount of heat produced with toluene was subtracted for correction. But there is still another possibility of explanation, namely the oxygen dissolved in the newly added sugar solution. It is quite probable that the dissolved oxygen would allow the weakened yeast to cause some oxidation, either of sugar or of other substances, and this would cause an increase of temperature. Any increase in temperature will be calculated by this method, as so much sugar fermented, and this would bring about a wrong conception. All oxygen dissolved will be readily used up by so much yeast during the first two hours, and after that, we find a normal fermentation. This heat from oxidation is too small to influence the data for an actively fermenting yeast, but becomes evident when the fermenting capacity is greatly reduced.

The average rate of fermentation showed no direct relation to the plate count, nor to the nitrogen content of the yeast, nor to the amount of alcohol formed.

It seems justified from the above data, to assume that the decrease of the rate of fermentation is practically due to the accu-

mulation of fermentation products, as far as the alcoholic fermentation is concerned.

TABLE 6

Rate of fermentation of yeast in sugar solution + alcohol

(The numbers are millimeters of mercury column per minute)

TIME AFTER MIXING YEAST AND SUGAR SOLUTION		ALCOHOL IN SOLUTION				
		0 per cent	1.37 per cent	2.74 per cent	4.11 per cent	5.48 per cent
hours	*minutes*					
	5–10	12.8	12.0	9.4	8.2	6.8
	10–15	12.8	11.6	10.4	9.0	7.2
	15–20	13.8	11.2	10.2	9.2	7.6
	20–25	14.2	12.2	12.4	10.0	8.4
	45–50		11.6	11.0		7.2
	50–55	15.4	13.0	12.4	8.2	8.6
	55–60	15.8	13.6	12.6	9.6	9.4
4	10–15	14.8	13.8	9.2	9.6	
	15–20	17.0	15.0	12.4	11.2	8.0
	20–25	17.4	15.4	13.6	12.0	9.4
Average rates......		14.8	12.9	11.4	9.7	8.1
Relative rates......		100	87.4	76.6	65.3	54.5

TABLE 7

Rates of fermentation as influenced by added alcohol

EXPERIMENT II			EXPERIMENT III			EXPERIMENT IV		
Alcohol in solution	Mercury per minute	Relative rates	Alcohol in solution	Mercury per minute	Relative rates	Alcohol in solution	Mercury per minute	Relative rates
per cent	*mm.*		*per cent*	*mm.*		*per cent*	*mm.*	
0	6.58	100.0	0	8.20	100.0	0	6.10	100.0
4.93	3.95	60.0	9.7	2.41	29.4	4.77	4.28	70.4
7.40	2.52	38.3	12.15	1.10	13.4	9.55	1.48	24.3
9.86	1.79	27.2	13.35	1.23	15.0	11.92	0.96	15.7
12.5	0.47	7.1				13.12	0.87	14.3
						14.31	0.74	12.1

The author could verify these data of Rubner's by determining the rate of fermentation of yeast with addition of definite amounts of alcohol by means of the "fermentometer" described in a previous paper. The rate is measured by the increase of pressure caused

through the production of carbon dioxide by yeast in sugar solutions. It is recorded here in millimeters of mercury column per minute. Ordinarily, the measurements are taken in intervals of five to ten minutes, but are calculated in millimeters per minute for comparative purposes.

Of the 4 experiments made, only one shall be given here in detail.

The average rates of the other 3 experiments and their relative rates are shown in table 7.
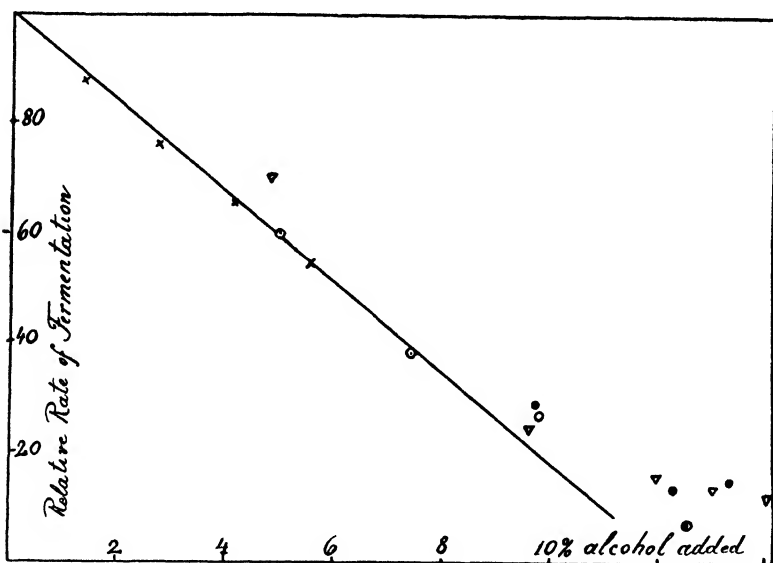
FIG. 1. RATE OF ALCOHOLIC FERMENTATION IN THE PRESENCE OF ALCOHOL

If the percentage decrease of rate of fermentation equals the concentration of alcohol present, there must be a straight line relationship between the relative values. Figure 1 shows that this is actually the case until very high values are reached. It seems that from 12 per cent alcohol on, there is a very slow yet noticeable rate of fermentation which is almost as high at 12 per cent as at 14.5 per cent.

It would be of interest to see how other fermentations fit into the above mentioned formula. Very few data are available con-

TABLE 8

Souring of milk by strain II of Strept. lactis

hours	N/10 NaOH FOR 100 CULTURE cc.	PLATE COUNT	Y million cells per cc.	$K = \frac{1}{ty} \log \frac{L}{L-x}$
0	16.5	38,000		
9	17.25	19,600,000		
12	18.5	113,000,000	66	12.9×10^{-12}
15	26.0	435,000,000	274	16.3×10^{-12}
18	45.75	1,135,000,000	787	18.6×10^{-12}
21	64.75	1,370,000,000	1,250	19.5×10^{-12}
24	69.75	1,550,000,000	1,490	22.0×10^{-12}
27	72.25	1,345,000,000	1,550	22.4×10^{-12}
30	74.75	1,300,000,000	1,550	23.2×10^{-12}
36	76.25	1,600,000,000	1,550	22.0×10^{-12}
L	80			
0	16.5	38		
15	17.0	4,800,000		
18	17.75	43,000,000	23.9	26.2×10^{-12}
21	19.50	176,000,000	109.5	52.4×10^{-12}
24	32.25	685,000.000	430.5	21.7×10^{-12}
27	54.00	1,235,000,000	960.0	18.9×10^{-12}
30	70.75	1,515,000,000	1,375.0	22.7×10^{-12}
33	75.00	1,390,000,000	1,452.5	25.6×10^{-12}
36	75.05	1,750,000,000	1,570.0	21.3×10^{-12}
Average.				23.1×10^{-12}

TABLE 9

Acid formation in neutralized milk culture

TIME AFTER NEU-TRALIZA-TION	A			B		
	N/10 NaOH	Plate count	K'	N/10	Plate count	K'
hours	cc.			cc.		
0	10.75	1,785,000,000		12.25	1,190,000,000	
2	36.25		63.7×10^{-12}	33.5		64.1×10^{-12}
4	58.50	3,190,000,000	62.9	53.75	2,975,000,000	53.3
6	66.0		64.2	63.75		58.6
8	68.0	3,245,000,000	59.5	69.25	3,285,000,000	75.0
24	69.5		27.7	69.0		23.5

cerning fermentations where the products as well as the numbers of cells have been determined at frequent intervals. Aside from the lactic fermentation, no data are known to the author. On the lactic fermentation, several series of experiments by the author (Rahn, 1911) and by Baker, Brew and Conn (1919) are available.

The equation developed for the alcoholic fermentation has to be changed slightly for the lactic fermentation because in the latter, the inhibiting product, the lactic acid, equals the amount of sugar disappearing. Therefore, we find

$$-\frac{dx}{dt} = k \cdot Y \cdot \left(1 - \frac{\text{acid}}{\text{limit}}\right) = kY\left(1 - \frac{x}{L}\right) = \frac{kY}{L}(L - x)$$

Integrated, this expression changes to

$$K = \frac{1}{t \cdot Y} \ln \frac{L}{L - x}$$

In this case, we have the disadvantage of Y, i.e., the number of acting cells, not being constant. We can count the numbers of cells, but we have no accurate way of calculating the average of the cells. In table 8 the number of cells is calculated as the average of the last two counts of Rahn's data. This gives no very good constants for the fermentation, but the tendency for a distinct average is quite evident.

The same strain was further tested in duplicate for its fermentation in a thirty-six-hour-old milk culture which had been neutralized and which had titrated 80 cc. N/10 alkali for 100 cc. The limiting acidity is here less than 80, probably between 69.0 or 70 cc N/10 NaOH. The constants calculated for $L = 70$ show a decrease in the last values, indicating that L is less than 70.

The data of Baker, Brew and Conn contain the hydrogen ion concentration as well as the titrated lactic acid. This gives two possibilities of calculating the fermentation constants. Their experiment I cannot be used because it is not possible to estimate the final acidity. Experiment II is given in table 10. The k values for fifteen hours are too high in both cases because the

TABLE 10

Acid formation by a pure culture of Strept. lactis in milk

hours	n/10 NaOH in 100 culture	HYDROGEN ION CONCENTRATION	MICROSCOPIC COUNT	Y	FERMENTATION CONSTANTS FROM	
					Titrated acidity	pH
	cc.					
0	0	3.2×10^{-7}	3,000			
15	1.0	3.7	35,000,000	17,500,000	$14\ 7 \times 10^{-12}$	$8\ 27 \times 10^{-12}$
16	2.2	4 1	115,000,000	75,000,000	7.88	2.17
17	3.5	4.9	205,000,000	160,000,000	5.65	1.11
18	5.3	6.2	395,000,000	300,000,000	4.38	0.80
19	9.2	10.2	645,000,000	520,000,000	4.31	0.70
20	15.0	10.8	1,120,000,000	882,000,000	4.08	0.42
21	23.7	35.0	1,430,000,000	1,275,000,000	4.53	0.84
22	36.2	66	2,400,000,000	1,915,000,000	4.70	1.06
23	55.8	190	3,600,000,000	3,000,000,000	5.36	2.32
24	64.0	270	2,800,000,000	3,600,000,000	5.40	2.54
40	83.5	480	4,800,000,000	4,800,000,000	4.26	2.77
64	97.3	680	2,550,000,000			

TABLE 11
Fermentation by Lactobacillus acidophilus in milk + lactic acid

| | PER CENT OF LACTIC ACID TITRATED | | | | | | | | | | |
	Start	½ hour	1 hour	1⅓ hours	2 hours	3½ hours	4½ hours	5½ hours	17 hours	24 hours	41 hours
A	0.16	0.19	0.20	0.23	0.25	0.30	0.34	0.37	0.74	0.80	0.92
B	0.25	0.28	0.29	0.31	0.32	0.38	0.42	0.45	0.77	0.79	0.94
C	0.35	0.36	0.38	0.38	0.41	0.47	0.50	0.53	0.78	0.80	0.93
D	0.44	0.45	0.46	0.47	0.49	0.52	0.57	0.58	0.80	0.80	0.90
E	0.54	0.55	0.55	0.56	0.57	0.62	0.63	0.64	0.79	0.81	0.89
F	0.63	0.63	0.65	0.63	0.64	0.68	0.68	0.69	0.79	0.81	0.86
G	0.73	0.73	0.74	0.74	0.74		0.76	0.77	0.83	0.80	0.81
H	0.80	0.81	0.82	0.83	0.82	0.81	0.82	0.84	0.84	0.81	0.82
I	0.89	0.89	0.89	0.90	0.90	0.90	0.91	0.90	0.91	0.89	0.88
J	0.90	0.98	0.98	0.99	0.99	0.98	0.98	0.99	0.98	0.96	0.95
	INCREASES PER HALF HOUR PERIOD										
A		0.03	0.01	0.03	0.02	0.017	0.020	0.015	0.016	0.0043	0.0035
B		0.03	0.01	0.02	0.01	0.020	0.020	0.015	0.014	0.0014	0.0044
C		0.01	0.02	0	0.03	0.020	0.015	0.015	0.011	0.0014	0.0038
D		0.01	0.01	0.01	0.02	0.010	0.025	0.005	0.0096	0	0.0030
E		0.01	0	0	0.01	0.017	0.005	0.005	0.0065	0.0014	0.0024
F		0	0	0	0.01	0.013	0	0.005	0.0043	0	0.0015
G		0	0	0.01	0	0	0.010	0.005	0.0013	0	0.0003
H		0.01	0	0	0	0	0	0	0	0	0
I		0	0	0	0	0	0	0	0	0	0
J		0	0	0	0	0	0	0	0	0	0

TABLE 12
Rate of fermentation per hour

By 420,000,000 cells of L. acidophilus per cubic centimeter

	INITIAL ACIDITY	RATE OF FERMENTATION IN PER CENT LACTIC ACID		INITIAL ACIDITY	RATE OF FERMENTATION IN PER CENT LACTIC ACID
	per cent	*per cent*		*per cent*	*per cent*
A	0.16	0.045	F	0.63	0.0065
B	0.25	0.0342	G	0.73	0.0054
C	0.35	0.0288	H	0.80	0.0018
D	0.44	0.0216	I	0.89	0
E	0.54	0.0162	J	0.90	0

average number of bacteria, Y, was too low. Aside from this, the uniformity of the fermentation constants computed with liberated acidity is quite satisfactory, while there is much more shifting if the calculation is based on H ion concentration.

These results could be verified by the author owing to the kindness of Dr. H. A. Cheplin of the Acidophilus Laboratory of the H. K. Mulford Company of Philadelphia. The author obtained a sufficient amount of pure living cells of *Lactobacillus acidophilus*
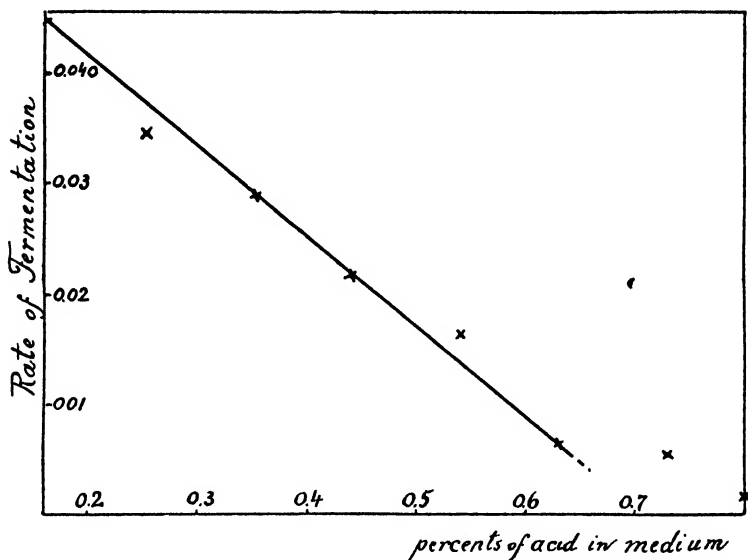
FIG. 2. RATE OF LACTIC ACID FERMENTATION IN THE PRESENCE OF LACTIC ACID

to add a large amount of it to milk, more bacteria than would normally grow in it. In this way, multiplication was eliminated, and the amounts of acid formed were all directly comparable.

Ten flasks of sterile milk were prepared, each containing 300 cc. of milk, to which 30 cc. of water was added containing sufficient lactic acid to increase the acidity of the milk by 0.1, 0.2, 0.3 per cent, etc. All flasks stood in the incubator over night before being inoculated each with 10 cc. of a suspension of acidophilus bacteria. Titration started at once, at first in half hour intervals, later in longer intervals, to obtain a good conception of the rate

of fermentation.   The milk contained 420,000,000 cells of *Lact. acidophilus* per cubic centimeter.

The increase in acidity, or the rate of fermentation, is not determined so very accurately, but by taking the average of the first two hours, the amounts of acid produced are still too small to reduce the rate of fermentation noticeably, and yet are large enough to be measured with a fair degree of accuracy.   Computing the average rate per hour, we obtain the results given in table 12.   If the rates are plotted against the initial acidity, a straightline relation is evident (fig. 2).   As in the alcoholic fermentation, there seems to be a tendency for a very slow fermentation when the straight-line extrapolation would indicate no fermentation at all.

SUMMARY

The retardation of a fermentation by its own products has been expressed in a simple formula on the assumption that the decrease of the rate of fermentation is proportional to the concentration of products divided by the limiting concentration; in other words, the decrease is proportional to the percentage of total possible products already formed.

This assumption leads to a formula for a "fermentation constant" representing the rate of fermentation corrected for any retardation by its own products.   The computation of this constant from available data on the alcoholic and lactic fermentations gave values of sufficient constancy to consider them a proof of the general principle involved.

Our experiments also prove that a straight-line relationship exists between the rate of fermentation and the amount of fermentation product added to the culture, in alcoholic as well as in lactic fermentation.

If the added fermentation products are as high as the limiting concentration, there still seems to take place a slow but noticeable fermentation provided that the number of fermenting cells is very large.

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THE ONTOGENY OF AN ORGANISM ISOLATED FROM MALIGNANT TUMORS

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During the course of investigations on malignant tumors an organism was isolated which, with its complex developmental history, defies definite classification. After four years of intensive study the conclusion has been reached by the authors that, though this organism may bear no relation to the production or development of tumors, a statistical presentation of single cell culture studies on eighteen strains might assist in bringing about some crystallization of ideas propagated through the great variety of contributions dealing with cultures from malignant tumors, as well as those relating to the morphology of more easily stabilized organisms.

SOURCE AND METHOD OF OBTAINING PURE CULTURES

Blocks were removed from the fresh tumor mass, using a rigidly controlled septic technic. Both malignant and non-malignant tumors were cultured. The entire tumor mass was first seared with a hot scalpel. The removed blocks were then cut into small bits with sterile scisssors and planted in a semi-solid medium (beef heart 500 grams; NaCl 5 grams; peptone 15 grams; agar 5 grams; K_2HPO_4 1 gram; and 2 eggs per liter of water) as well as in a liquid medium (containing in the place of the agar in the semi-solid medium 0.5 per cent gelatin). These media were prepared as are the usual hormone media. Both aerobic and anaerobic methods, using tubes overlaid with paraffin oil, were used.

The tubes and plates were incubated at 37°C. and examined daily. The type of tumor (i.e., whether it was an adeno or

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scirrhous type) seemed greatly to influence the time required for the growth to develop. This time period varied from three days to three weeks. Upon obtaining the initial growth from the tumor mass it was repeatedly streaked on the same medium, single colonies being replanted. As soon as the organism grew with less reluctance on a solid medium, single cell cultures were prepared using Orskov's single cell culture technic. Usually a period of a month was consumed in getting the culture to this stage, since the intial growth developed slowly and the subsequent transplants often failed to grow. The cultural and morphological studies which follow must be considered with these facts in mind, since slow acclimatization to a new environment seems to affect the reactions of this organism.

CONTROLS

The beef heart media were sterilized by heating on three successive days to 100°C. for one hour in the Arnold sterilizer. All of the other media were sterilized at 17 pounds steam pressure for twenty minutes in the autoclave. The media were incubated at 37°C. for not less than three days, and usually for one week. Uninoculated control tubes and plates of the media were always incubated with the inoculated ones throughout the whole period of incubation. At no time was the organism obtained from the un-inoculated media or from non-malignant tumors.

After incubation, liquid media were examined by making smears of any sediment in the tubes. Likewise, cultures were regularly examined microscopically before inoculation, after the first day of incubation and at the end of the incubation period. It was due to this routine performance that results were soon obtained which were at first perplexing but later intriguing because of the consistent and convincing evidence of the greatly varying forms of the organism—an organism obtained from a single cell.

MORPHOLOGY

Several methods were used to determine the ability of the organism to assume varied forms. Hanging drops, inoculated with the organism, were incubated for various periods and the growth was
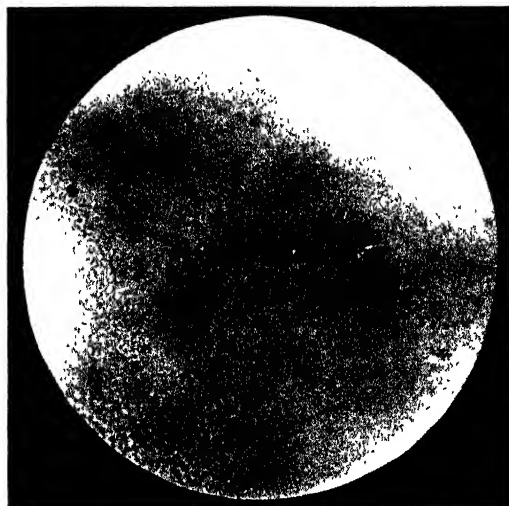
FIG. 1. COCCO-BACILLI FROM ANAEROBIC CULTURE ASCITIC FLUID.
(TISSUE 28)
Magnification 600 diameters
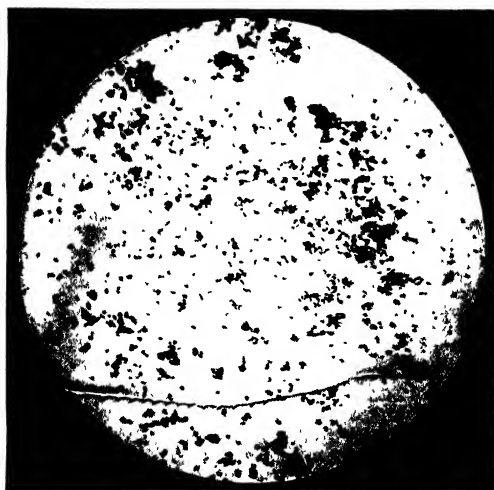
FIG 2 POINTED ROD—FROM SOLID MEDIUM
Magnification 600
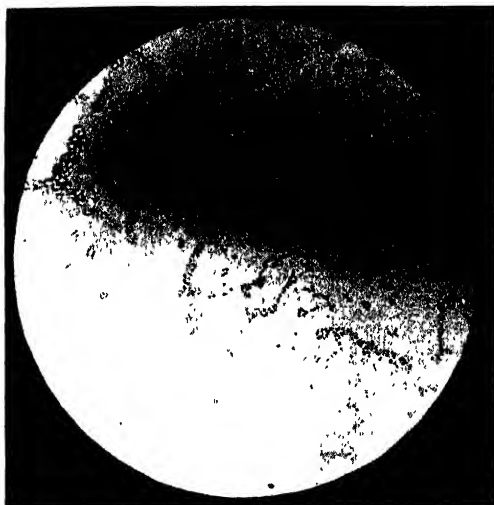
FIG. 3. COCCI (STARCH PLATE)
Magnification 600
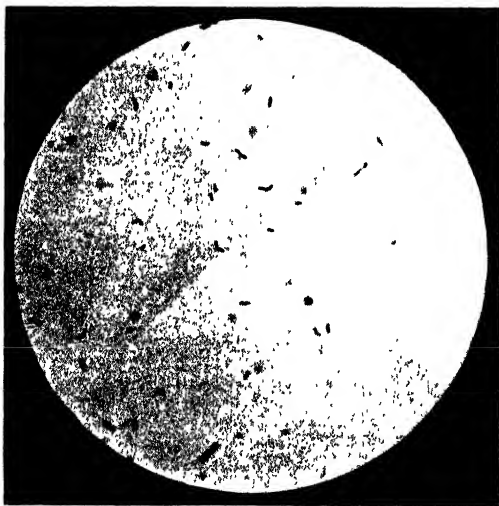
FIG. 4. CURVED ROD FROM FORTY-EIGHT-HOURS LACTOSE BROTH CULTURE

FIG. 5. BRANCHING FORMS (ONE-WEEK-OLD BOUILLON CULTURES)

FIG. 6. LARGE OVAL COCCI, GLOBULES OR SPORE SACS AND SMALL COCCO-BACILLI

Minute cocco-bacillus
(fig. 1)

Pointed rod ←--- ---------→ Curved rod   *Motile coccus* (fig. 3)
(fig. 2)   (fig. 4)

Branching forms
(fig. 5)

Gonidia

Small   Small
bacillus   coccus

Large oval
coccus   From Berkefeld filtrate
(fig. 6)   of cultures contain-
                ing gonidia, small
                cocci and rods
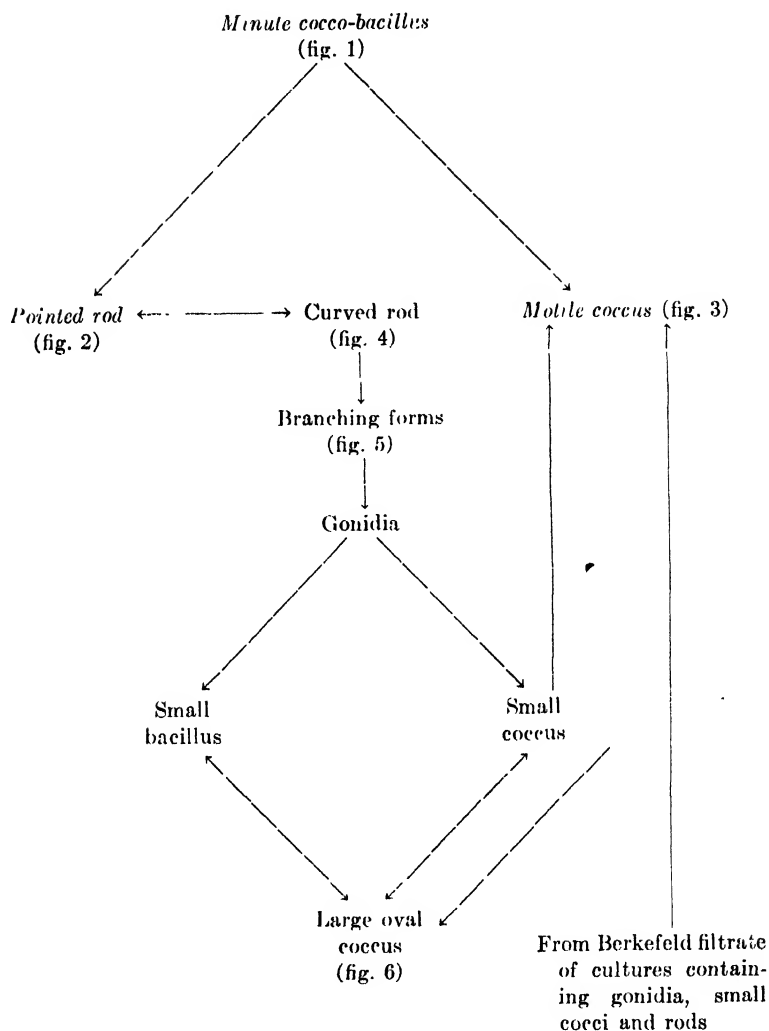
FIG. 7

then stained either by Nakanishi's intravital staining method or in the usual fixed smears. The method of rapid transfer at intervals to various media, with subsequent microscopic examination at the times of transfer, was also used.

The various morphological forms exhibited during development

Fig. 7A. Camera Lucida Drawings Corresponding in Position to Forms Named in Fig. 7

under artificial cultural conditions can be said to belong to either: (1) a more or less permanent phase in which any one of three forms will retain its shape indefinitely under controlled conditions and in which the method of multiplication is by fission, or (2) a transient phase in which the organism, through new modes of development, acclimatizes itself to adverse and new conditions and rejuvenates the strain. The three distinct morphological forms found in the first phase are:

1. A minute cocco-bacillus varying from 0.23 to 0.3 micron in width and from 0.35 to 0.6 micron in length (fig. 1).

2. A slightly pointed rod varying from 0.8 to 1.5 microns in length and from 0.3 to 0.4 micron in width (fig. 2).

3. A coccus varying in diameter from 0.35 to 1.3 microns (fig. 3).

Forms of the second phase are:

1. A long curved rod 0.6 to 0.8 micron in width and 1,5 to 3.0 microns in length (fig. 4).

2. Branching forms 0.4 to 1.0 micron in diameter and 3.0 to 8.0 microns in length (fig. 5).

3. Gonidia resembling minute cocci about 0.2 micron in diameter.

4. Enlarged oval cocci (possibly spore sacs) (fig. 6).

Diagrammatically the inter-relationships of these forms are shown in figure 7. The early spiral and filamentous forms which usually grow out at first are seldom retained upon cultivation. They fragment into the minute cocco-bacillus. The methods used so far, i.e., reduced oxygen tension, variation of pH, etc., have yielded no knowledge as to the cultural conditions requisite for this form to persist.

The minute cocco-bacillus (fig. 1) may be considered the stabilized form which the organism assumes when it grows and multiplies under *anaerobic* conditions. In sera containing pieces of brain tissue or even meat infusion media overlaid with paraffin oil this form grows, producing a very finely granular turbidity. When grown according to Wright's technic it also shows this form. After a week's incubation of the minute form on Russell's agar large oval cocci appear (fig. 6).

The small to medium pointed rod (fig. 2) is the form found growing from the minute cocco-bacillus as well as from the small bacilli which develop from gonidia.   The organism retains this form on solid medium indefinitely under conditions of daily transplantation.   When it is transplanted to broth or to tubes containing a large amount of water of condensation there is, after forty-eight to seventy-two hours, a gradual enlargement, the rod becomes hooked and curved, and, after a week, branching forms appear (fig. 5).   These forms are beaded in appearance at first, developing vacuoles with simultaneous appearance of gonidia in the medium.

The coccus form cannot be distinguished from a staphylococcus culture.   If, however, acid is produced in the medium the coccus gradually changes to small cocci and rods which, if transplanted to broth, will develop into the small or medium rod.   On the other hand, if the original cocci are cultured on starch agar or other solid media in which there is no pH change, they multiply by fission and retain the coccus form.

It has been found necessary to change the environment radically after the organism has remained in a dormant condition during storage.   For example, if stored on agar as the minute coccobacillus, it is advisable to transplant to sugar broth rather than to agar again.   In most cases growth will fail to occur on the latter.   In the former, however, the new phase of development is entered, with resulting invigorating of the organism.

GRAM CHARACTER

Various methods were used as checks against one another. The classical Gram technic, Burke's modification, as well as those of Hucker, of Sterling, and of the authors in which 0.5 per cent potassium dichromate applied for one minute was used as mordant and acetone as decolorizer, were employed.   The curved rod form from aerobic cultures, and the coccus from broth and agar cultures are strongly Gram-positive.   The small cocco-bacillus from anaerobic cultures, as well as all of the smaller forms such as the gonidia and the minute bacilli, are Gram-negative.

The organism is not acid fast.   At times it assumes a Gram-

positive stippled appearing center surrounded by a Gram-negative wall.   With methylene blue it shows a beaded appearance.

CAPSULE

Using the Hiss method for capsule staining, the organism, when grown on blood agar slants, will show the presence of capsules.

MOTILITY

All forms, minute cocco-bacillus, rod, coccus and gonidium, of all the strains isolated exhibit motility.   When the water of condensation from cultures on the beef heart medium is examined, the rods exhibit the greatest motility.   However, strains which have been subcultured for three years have lost their motility.

When stained by Fontana's silver nitrate spirochaete method, flagella, with lophotrichiate attachment can be demonstrated on the rods.   Flagella on the cocci have not been successfully demonstrated.   The presence of flagella could not be demonstrated by either Loeffler's or Shunk's stain.

SPORE PRODUCTION

The medium sized rod grown for one week or more on beef extract agar will show spore-like bodies which remain unstained when the smear is stained by the Gram technic.   If growth from such a culture is suspended in sterile water and heated for ten minutes at 85°C. and then transplanted to the semi-solid medium, growth of the pointed rod is obtained.   By means of Dorner's method the round spores of the organism are stained red.

THE GENERAL BIOLOGICAL CHARACTERISTICS

Appearance of aerobic growth in broth.   In liquid media, in which the organism develops easily, as in glucose broth and gelatin beef heart medium, there is after twenty-four hours a slight clouding, which increases within seventy-two hours, so that, macroscopically, the growth appears as a strong, finely granular turbidity of the broth, there being no pellicle formed and only a moderate viscid sediment after 72 hours' incubation.

Appearance of aerobic growth on agar. On beef extract agar adjusted to a pH between the limits 6.8 and 8, and containing 1.5 per cent agar, the growth, after twenty-four hours' incubation at 37°C. is sparse, colorless but glistening, beaded and scarcely perceptible. After forty-eight hours the moderate spreading growth is raised, cream-yellow and somewhat viscid, but the medium remains unchanged. On beef heart 0.8 per cent agar slants the growth is much more rapid, viscid and spreading, and usually becomes wrinkled within ninety-six hours of incubation.

Bacterial action upon erythrocytes. The organism in its various forms was streaked on blood agar plates and tubes. The plates were incubated at 37°C. for from one to three days. The organism belongs to the group known as "indifferent," producing no change in the hemoglobin or erythrocytes.

ODOR

The organism, when newly isolated and grown on beef heart medium, produces an amine or fish-like odor. In such agar cultures, as well as in broth cultures, there is a gradual deposition of crystals with a gradual disappearance of the amine odor.

GELATIN LIQUEFACTION

The growth of the organism on plain gelatin at a pH of 7.4 to 7.6 was very scant. Nutrient gelatin adjusted to a pH of 7.4 was found to give sufficient growth at room temperature. Cultures were incubated both at room temperature and at 37°C. for periods lasting from ten days to six weeks, and they were then placed in the refrigerator to test for liquefaction. In the gelatin stab cultures the growth is best at the top, the line of puncture appearing slightly beaded. Slow liquefaction takes place at room temperature after ten days, but the liquefaction at no time extends far from the point of inoculation.

OXYGEN REQUIREMENT

After the organism has adjusted itself to a saprophytic existence it shows a distinct tendency to grow on the surface and upper

layers of glucose, sucrose, and lactose shake cultures, producing acid but no gas. When the agar is not too stiff, small colonies grow throughout such cultures. The organism grows with the production of acid in lactose broth covered with paraffin oil. It is a *facultative anaerobe*.

TEMPERATURE REQUIREMENTS

The original growth of the organism from tumors has never been obtained at temperatures below 37°C. After it has accustomed itself to artificial conditions it produces distinct turbidity in twenty-four hours at 37°C. in glucose broth, but not at 25°C. On sugar agar slants the growth is sparse after twenty-four hours at 37°C., likewise it is sparse after forty-eight hours at 25°C.

At room temperature the growth of the organism is only slight. The optimum temperature requirement is 37°C., though it will grow at 40°C.

RELATION TO REACTION OF MEDIUM

Beef extract agar and beef extract broth were titrated with HCl and NaOH, and the pH was colorimetrically determined in one series for testing the effect of the reaction of the medium. In another series the pH was adjusted by means of phosphate buffers. The organism in its coccus form from a twenty-four-hour agar slant, in the large curved rod form from twenty-four-hour lactose broth tubes, and in the minute cocco-bacillus form from blood agar slants, was inoculated into tubes of media with pH ranging from 4.8 to 9.2. Growth does not occur during a week's incubation at 37°C. at pH values below 5.6 in broth cultures, though agar cultures at that pH will show a dry sparse white growth which, examined microscopically, is found to consist of swollen, club-shaped, and branching forms. Agar adjusted to a pH of 8.6 will permit growth.

The cocco-bacillus fails to grow at that pH in broth while the curved rod will grow at a pH as high as 9.

The optimum pH is about 7.4, though there is no evidence that the rate of growth is influenced to any great extent by change in pH through the range 7.0 to 7.8.

CARBOHYDRATE FERMENTATION

In the tables results of the studies on carbohydrate fermentation for various incubation periods at 37°C. are given.   Cultures were

TABLE 1A

Rod form.   Beef extract broth containing brom cresol purple and 1 per cent carbohydrate

CARBOHYDRATE	INCUBATION PERIOD IN DAYS							
	1	2	3	4	5	6	7	14
Glucose	−	?	+	+	+	+	+	+
Lactose	−	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	+	+	+
Sucrose	−	−	±	±	±	+	+	+
Mannitol	−	−	−	−	−	−	−	−
Glycerol	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−
Lacvulose	−	−	±	+	+	+	+	+
Inulin	−	−	−	−	−	−	−	−
Dextrin	−	−	−	?	?	±	±	+

Gas was not formed at any time.   Control tubes were incubated and used for comparison with the inoculated tubes.

TABLE 1B

Coccus form.   Media the same as in table 1A

CARBOHYDRATE	INCUBATION PERIOD IN DAYS							
	1	2	3	4	5	6	7	14
Glucose	−	±	+	+	+	+	+	+
Lactose	−	−	−	−	−	−	−	−
Maltose	+	+	+	+	+	+	+	+
Sucrose	−	−	±	+	+	+	+	+
Mannitol.	−	−	−	−	−	−	−	+
Glycerol	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−
Laevulose	−	−	±	+	+	+	+	+
Inulin.	−	−	−	−	−	−	−	−
Dextrin	−	−	−	±	+	+	+	+

used which, at the time of inoculation consisted of one of two distinct morphological forms.   A rod form came from an eighteen-

hour-broth culture and a coccus form from an eighteen-hour agar slant. Brom cresol purple was added to the various media. Mircroscopic examination of the tubes after twenty-four hours and at the end of the incubation period was made. The sugar

TABLE 1C

Rod form. Beef extract agar containing brom cresol purple and 1 per cent carbohydrate

CARBOHYDRATE	INCUBATION PERIOD IN DAYS							
	1	2	3	4	5	6	7	14
Glucose	+	+	+	+	+	+	+	+
Lactose...............	−	−	−	−	±	+	+	+
Maltose....;	−	+	+	+	+	+	+	+
Sucrose	−	−	±	+	+	+	+	+
Mannitol..	−	−	−	−	?	+	+	+
Glycerol	−	−	−	±	+	+	+	+
Salicin............	−	−	−	−	−	−	−	−
Laevulose	−	−	−	−	−	−	−	±
Inulin....	−	−	±	+	+	+	+	+
Dextrin	−	−	+	+	+	+	+	+

TABLE 1D

Coccus form. Media same as in table 1C

CARBOHYDRATE	INCUBATION PERIOD IN DAYS							
	1	2	3	4	5	6	7	14
Glucose	±	+	+	+	+	+	+	+
Lactose	−	±	±	±	±	±	±	±
Maltose	+	+	+	+	+	+	+	+
Sucrose	−	±	+	+	+	+	+	+
Mannitol.	−	−	−	−	−	−	−	−
Glycerol.	−	−	±	+	+	+	+	+
Laevulose	−	−	−	+	+	+	+	+
Inulin.	−	−	±	+	+	+	+	+
Dextrin	−	−	+	+	+	+	+	+

under observation was added after sterilization and all tubes were incubated, as sterility test, for periods of not less than three days. In the tables + indicates distinct acid formation, ± faint acid formation and − indicates no change in pH.

In beef extract broth containing brom cresol purple and 1 per cent carbohydrate sufficient acid is formed to detect fermentation from glucose, maltose, sucrose, laevulose and dextrin.   After three days of incubation all broth cultures show almost uniformly the curved rod form.

At the end of one week's incubation all cultures show the coccus form.   In beef extract agar the rod and coccus forms ferment glucose, lactose, maltose, sucrose, glycerol, laevulose and inulin with the production of sufficient acid to be detected with brom cresol purple.   The coccus form fails to ferment mannitol on agar.

TABLE 2

Results same for both forms.   Synthetic medium with 1 per cent carbohydrate, brom cresol purple and cresol red

CARBOHYDRATE	INCUBATION PERIOD IN DAYS							
	1	2	3	4	5	6	7	14
Lactose	−	−	−	±	±	±	±	±
Glycerol	−	−	−	±	±	±	±	±
Mannitol	−	±	±	+	+	+	+	+
Inulin	−	−	−	−	−	−	−	−
Dextrin	−	+	+	+	+	+	+	+

To further test the fermentation of those carbohydrates from which only small amounts of acid were formed a synthetic broth (prepared according to the directions on page 11b of the Manual of Methods) was used to which was added 1 per cent of the carbohydrate and the two indicators brom cresol purple and cresol red.

The same results were obtained from both the rod and the coccus forms of the organism.   Though growth in the synthetic medium is never as great as in a peptone containing medium, the organism develops as a very delicate curved rod and slowly produces acid from those carbohydrates so marked in table 2.

The following is a brief summary of a large number of varied experiments on carbohydrate fermentation:

The organism readily ferments glucose, sucrose, maltose and laevulose.   Dextrin too is fermented by most strains.   Sufficient

acid is formed from these carbohydrates to permit its detection by means of brom cresol purple in the broth cultures.

Lactose, glycerol and mannitol are fermented with the production, in most cases, of enough acid to be detectable only in synthetic media, i.e., in the absence of peptone, either by means of brom cresol purple and cresol red or by electrometric measurement.

The organisms seem incapable of fermenting inulin and salicin in liquid media.   Raffinose is fermented in agar media by only two strains.

Acid is produced from lactose in anaerobic cultures more readily than in aerobic cultures.

The ability to ferment any of the sugars gradually decreases upon storage despite subsequent and frequent transplantation before testing.

Russell's double sugar agar gives, within forty-eight to seventy-two hours, distinct evidence of acid production.

ACID PRODUCTION IN MILK

Both litmus and brom cresol purple milks were used to determine acid production in milk.   Cultures were made under aerobic and partially anaerobic conditions, i.e., overlaid with paraffin oil, and incubated for periods of from one to ten days at room temperature and at 37°C.   At no time was a rennet or acid curd formed.   A faint acid reaction was often obtained, but not regularly, due possibly to poor growth in the milk medium and to non-sensitivity of the indicator to slight pH changes.

REDUCTION OF NITRATES

Twenty-four hour cultures were inoculated into 0.1 per cent KNO_3 beef extract agar as well as into the synthetic nitrate broth recommended for use on page B27 of the Manual of Methods for Pure Culture Study of Bacteria.   The results of these experiments are given in table 3.

These results show that the organism reduces nitrate to nitrite in both KNO_3 agar and broth, though no gas is formed.

CHROMOGENESIS

Color production varies from a cream to a distinct sulphur yellow when the organism is grown on beef extract agar medium and on potato slants.   On gelatin it develops only a pale yellow color.   Often during the first three days of growth on agar slants it is nearly white or cream colored, after which it suddenly produces a sulphur yellow color.   On blood agar slants it has a dull cream color, and on brom cresol purple sugar agar slants it is colorless.   On slants of Russell's agar, where it produces acid, it is colorless.

TABLE 3

FORM		KNO_3 BROTH					KNO_3 AGAR					SYNTHETIC MEDIUM*				
				Incubation period in days												
		1	2	4	7	10	1	2	4	7	10	1	2	4	7	10
Cocco-bacillus	Gas	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	KNO₂	−	−	±	+	+	−	−	±	+	+	−	−	−	?	±
Coccus	Gas	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	KNO₂	−	−	±	+	+	−	−	±	+	+	−	−	−	?	±
Rod	Gas	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	KNO₂	−	−	±	+	+	−	+	+	+	+	−	±	+	+	+
Control	Gas	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	KNO₂	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salmonella aertrycke	Gas	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	KNO₂	−	−	±			+	+	+	+	+	+	+	+	+	+

* The organism from tumors grows only very slightly in any synthetic medium.

HYDROLYSIS OF STARCH

One strain out of the eighteen exhibited some diastatic activity. All other strains gave consistently negative results.   Two-tenths per cent soluble starch agar plates were streaked with twenty-four-hour cultures, and as the organism grew slowly, the observations were made, after an incubation period of seven to ten days, by flooding the surface of the petri dishes with a saturated solution of iodine in 50 per cent alcohol.

INDOL PRODUCTION

Three kinds of media were used to test the formation of indol. Nutrient broth containing chloroform was digested with trypsin at 37°C. for twenty-four hours, and then filtered and sterilized in tubes. A 1 per cent Witte's peptone solution was tested, but the organism was unable to grow in this solution except in rare

TABLE 4

FORM	MEDIUM: TRYPSINIZED BOUILLON TEST: VANILLIN TEST				MEDIUM: 1 PER CENT DIFCO PEPTONE BEEF EXTRACT BROTH TEST: ETHER AND EHRLICH'S REAGENT			
	Incubation period in days							
	2	4	7	10	2	4	7	10
Cocco-bacillus	−	−	±	+	−	−	−	±
Coccus....................	−	±	±	+	−	−−	−	±
Rod........................	−	±	±	+	−	−	−	±
Bacillus coli...	+	+	+	+	+	+	+	+
Control...................	−	−	−	−	−	−	−	−

TABLE 5

FORM	KLIGLER'S LEAD ACETATE AGAR				WILSON'S FERRIC CHLORIDE AGAR				MODIFIED LEAD ACETATE MEDIUM			
	Incubation period in days											
	1	2	5	10	1	2	5	10	1	2	5	10
Cocco-bacillus..	−	−	−	−	−	−	−	−	−	−	+	+
Coccus.................	−	−	−	−	−	−	−	−	−	±	+	+
Rod...	−	−	−	+	−	−	−	−	−	+	+	+
Bacillus coli	−	−	−	−	−	−	−	−	−	−	−	−
Bacillus subtilis	−	−	+	+	−	+	+	+	−	+	+	+
Control	−	−	−	−	−	−	−	−	−	−	−	−

instances, so that all tubes, when tested by the Ehrlich-Böhme technic, were negative, though the cultures of Bacillus coli gave positive tests for indol formation by this technic. The third medium used was 1 per cent Difco peptone beef extract broth. The results are given in table 4.

Indol production is not a constant nor a pronounced characteristic of the organism.

PRODUCTION OF HYDROGEN SULPHIDE

For the determination of hydrogen sulphide production three different media were used involving three different methods: Kligler's lead acetate method, Wilson's agar method, and finally a modification of Kligler's method using 30 grams of peptone and only 8 grams of agar and adjusting the medium to a pH of 7.8. The media were incubated for three days, and, after inoculation and incubation for the stated periods, microscopic examination of the growth was made.   Results are given in table 5.   The organism produces hydrogen sulphide in a very soft agar containing an increased amount of peptone.

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THE EFFECTS OF CERTAIN CHEMICAL COMPOUNDS UPON THE COURSE OF GAS PRODUCTION BY BAKER'S YEAST[1]

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INTRODUCTION

During a recent investigation of a method for standardizing antiseptics on the basis of their ability to inhibit fermentation by yeast, observations were made on the effects of chemical compounds upon the course of gas production by these organisms. The method used was a modification of the technic employed by Pilcher and Sollmann (1922–1923) and by Peterson (1926) whose work was based upon Dreser's (1917) suggestion that the inhibition of gas production by certain compounds acting upon yeast could be used as a measure of the antiseptic efficiency of these compounds. As the apparatus used by these authors permitted large and undetermined amounts of the gas to escape around the outside of the fermentation tubes, it was necessary to change their procedure by employing a vessel which would collect all of the gas. The introduction of a simple gasometer at once enlarged the scope of the method, making it a more accurate means of determining the inhibiting effect of antiseptics, and at the same time converting it into a means for obtaining information in regard to some phases of the biology of yeast. A comparison of antiseptics on the basis of the results obtained through the collection of the total gas evolved has been published elsewhere (Branham, 1929). The chief purpose of this paper is to report

observations of more general physiological interest in connection with the action of chemicals upon the rate and volume of gas production by *Saccharomyces cerevisiae* in solutions of sucrose.

A group of workers at Greifswald in 1884–1888 were among the first, after Pasteur, to study the effects of chemicals upon yeast fermentation. They observed the rate and amount of carbon dioxide production in mixtures of yeast cells and sugar solutions to which these substances had been added. Hoffmann (1884), Thol (1885), Gottbrecht (1886), and Schulz (1887, 1888) presented extensive observations made with thallium tartrate, mercuric chloride, iodine, bromine, arsenic acid, chromic acid, formic acid, and salicylic acid, showing that high dilutions caused a striking increase in fermentative activity, whereas carbon dioxide formation was markedly inhibited by greater concentrations. More recently Peterson (1926) found a stimulation of carbon dioxide production in high dilutions of a few of the mercury compounds with which he worked. Harden and Young (1911) found the same phenomenon in the action of arsenates and arsenites upon yeast juice. The enzymes in the juice are unaffected by many substances that are very toxic for the living cells, so that results obtained with this type of material are not consistently comparable in this respect with those obtained with whole yeast.

Joachimoglu (1922) was unable to confirm the observations of the Greifswald investigators in that he found that small amounts of mercuric chloride, phenol, and quinine did not stimulate fermentation in the mixtures of sugar and yeast which he used. He agreed that some substances apparently cause an increased activity in high dilutions, but did not consider that these observations could be generally applied.

Many other investigators have studied the toxicity of certain compounds for yeasts. Lindner and Grouven (1913) found mercuric chloride, fluorides, formalin, and antiformin to be regularly toxic for yeasts in all dilutions studied. Dreser (1917), Zerner and Hamburger (1921), and Pilcher and Sollmann (1922–1923) have used the inhibition of carbon dioxide production by yeasts

as a criterion for comparison in their studies of silver compounds. Euler and Emberg (1919) noted appreciable changes in the rate of growth and the cell composition of yeasts under the influence of various hydrogen ion concentrations. Somogyi (1921) found many organic and inorganic acids to have a harmful effect upon yeasts. Euler and Nilsson (1925) and Brown and Wikoff (1927a) have found hydrogen peroxide to prevent fermentation to a marked degree, and these latter workers also found (1927b) hexyl resorcinol powerfully toxic for yeasts. Myers (1927) found that certain volatile oils, particularly thymol, exerted a marked fungicidal action. Dann and Quastel (1928) have made a systematic investigation of the effects of a number of glucosides and polyhydric phenols on yeast fermentation. They found that allyl alcohol, acrylic acid, and phloroglucinol inhibited fermentation, both by the living cells and by zymin, whereas phlorizin inhibited zymin, but not living microörganisms.

The methods used to estimate the effect of the compounds upon the activity of yeasts have varied widely. Schulz (1887, 1888) measured the pressure of the evolved carbon dioxide by means of a mercury manometer attached to the apparatus which he used. Slator (1906, 1908) employed a method similar to that of Schulz. Euler and Lindner (1915) used a Meissl ventilation valve which allowed the carbon dioxide to escape, but retained the water vapor by causing the gas to bubble through sulphuric acid. The losses in weight were calculated as carbon dioxide evolved. Alwood (1908) adopted 'a similar apparatus which he used in the same way. Joachimoglu (1922), described an apparatus in which the amount of carbon dioxide produced was determined by weight. Dreser (1917) and Pilcher and Sollmann (1922–1923) collected the carbon dioxide in an inverted tube and then measured the column of gas in linear centimeters. Dann and Quastel (1928) measured the carbon dioxide in cubic centimeters by means of an apparatus originally devised by Tryhorn and Jessop, which, while accurate, seems too elaborate for general or large scale use. Lindner and Grouven (1913) used the killing power of a compound as a criterion. Zerner and Hamburger (1921) also used killing power, but determined it in

a different way.   Euler and Nilsson (1925) measured the total yeast and total sugar present in samples withdrawn from their test mixtures at definite intervals.   Similarly Balls and Brown (1925) and Brown and Wikoff (1927a, 1927b) made regularly timed estimations of total solid, total sugar, weight of yeast crop, and rate of inversion of sugar.   Somogyi (1921) determined the amount of sugar in the fermenting yeast-glucose-acid mixtures which he was investigating.   Quite different from these methods was that of Gutstein (1927) who made a microscopical study of the morphology of yeast cells treated with the dyes and salts of the heavy metals which he studied.

Interesting studies have been made of the chief factors which control the rate and amount of fermentation by yeasts.   By using a bottle connected with a manometer for measurement of gas, and making readings every five minutes, Slator (1906, 1908) found the velocity of fermentation to be directly proportional to the concentration of the yeast, and independent of the concentration of the sugar between 1 and 10 per cent.   Sucrose, glucose, and levulose were fermented at the same rate by the brewery yeasts which he used.   Slator (1913) stated that fermentation, as well as growth of cells, is logarithmic.   Tammann (1889) found that the course of fermentation, when observed from beginning to end, did not fall on a logarithmic curve, but more nearly approached a straight line.   Balls and Brown (1925) reported that yeast growth is logarithmic only when the weight of the total yeast is considered instead of the number of cells. Köhler (1920) considered the sugar concentration to be a controlling factor in growth velocity, and claimed that the processes, both of carbon dioxide production and growth, occurring in alcoholic fermentation follow a rhythm; i.e., an increased rate follows a decreased rate according to the variation in sugar and alcohol content of the medium.   The studies of Lindner and Grouven (1913) and of Zerner and Hamburger (1921) indicate that various yeasts show great differences in activity, as well as in resistance to toxic substances, and that there is a direct relation between the amount of each yeast used and the concentration of such substances required to kill or inhibit its action.

Joachimoglu (1922) called attention to the fact, doubtless noticed by many, that fermentation in a series of flasks, containing the same amount of the same mixture, may not be equal in degree or rate.

EXPERIMENTAL WORK

The apparatus used in the studies reported in this paper is illustrated in figure 1. The larger tube (*a*), graduated for 30 cc.,
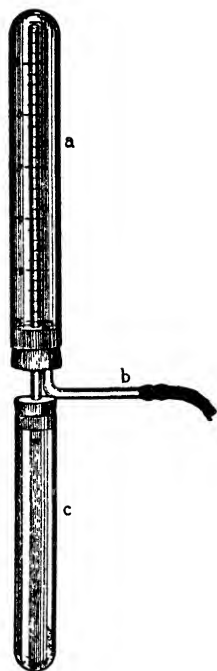
FIG. 1

is fitted with a two-hole rubber stopper carrying two pieces of glass tubing; one extending to the bottom of the test tube, and one, bent at right angles, which barely reaches the inner surface of the stopper. The larger graduated tube (*a*) is filled with water, inverted, and fitted by means of a second rubber stopper into the smaller tube (*c*) containing the yeast-sugar solution. Then the completed apparatus is placed in a water bath deep enough to cover the opening of (*b*). The amount of gas can be read in

cubic centimeters by means of the graduations on (a). Special copper racks were made for these simple gasometers so that numbers of them could be handled at one time.

Before attempting to observe the effects of chemicals upon fermentation it was necessary to determine the most suitable concentrations of yeast and sugar for regular use. With some proportions more gas was evolved than could be conveniently collected; with others, fermentation proceeded so slowly that it was difficult to determine the end-point and to keep other factors constant.

In order to find suitable proportions of yeast and sugar for satisfactory experimentation, various sets of 12 tubes each were set up with different combinations of yeast and sugar: Two, 4, 5, and 10 per cent suspensions of yeasts were used with 1, 2, 5, and 10 per cent solutions of cane sugar. Fermentation did not proceed at a uniform rate in all tubes containing the same amount of the same yeast and sugar mixture; i.e., the apparatus containing the greatest amount of gas after 15 minutes would not regularly be the one containing most after thirty or forty-five minutes, or one hour. But when fermentation was allowed to proceed to completion the total amount of carbon dioxide formed in each apparatus was found to be approximately equal. With 10 per cent yeast and 1 per cent sugar the reaction proceeded quickly and was practically finished within 1 hour. The total gas formed in these tubes was relatively uniform, and the volume was easily contained in gasometers of the size described. These proportions were used in all of the following studies.

The experiments were repeated with many samples of Fleischmann's yeast, including both one-pound bricks, containing 1 per cent starch as a binder, and the usual small cakes, with 2 per cent corn starch. Both glucose and sucrose were used; there was no appreciable difference in the results obtained, so that Merck's "Highest Purity—C.P." sucrose was used throughout the rest of this study.

Individual samples of yeast, through shipping and storage, necessarily vary in activity. Consequently, if the effect of different chemicals upon the course of gas production was to be

studied, it was obviously important to observe the normal course of carbon dioxide evolution with every sample of yeast used. As a matter of fact, not only with every sample of yeast, but every time that a test was made, a series of tubes containing only the 10 per cent yeast and 1 per cent sugar mixture was included. Besides serving as a check on the variability of the yeast used in each experiment, this obviated the necessity for making corrections for daily changes in barometric pressure which would be theoretically necessary for absolute accuracy.

There was always some residual air in the small tubes (c) containing the 10 cc. of yeast-sugar mixture. The temperature of the water bath caused this to expand and also caused much of the dissolved gases to separate out so that readings of 0.5 to 1.0 cc. were always obtained in the large tubes (a) whether fermentation had occurred or not. By taking preliminary readings after the first five minutes of incubation these amounts could be read directly and corrections made by subtracting them from the total amounts of gas formed. A "lag" of about eight minutes, which occurred before carbon dioxide began to be collected in tube (a), gave ample time for these preliminary readings to be made.

No corrections were made for dissolved carbon dioxide or for carbon dioxide pressure within the tubes. Since the gasometers were of approximately the same size, and uniform quantities of yeast and sugar were put into them, the dissolved carbon dioxide and carbon dioxide pressure may be considered as practically a constant in any given series of tubes. However this would not be true of other series, particularly if they contained varying dilutions of chemicals that would alter the pH of the medium, thereby changing the solubility and rate of liberation of carbon dioxide, since both of these factors are affected by the hydrogen ion concentration. The influence of hydrogen ion concentration should be taken into consideration when absolute accuracy is desired in comparing the action of different dilutions of such compounds.

An investigation of the effect of certain chemicals upon yeast fermentation was now begun. The materials studied were of

widely different composition, and were chosen with reference to the development of a satisfactory method for standardizing antiseptics reported in another paper (Branham, 1929). These compounds were as follows: 3 samples of mercuric chloride, 3 of mercurochrome, 3 of silver nitrate, 2 of phenol, lysol, tricresol, 2 creosote oil preparations, hexyl resorcinol, metaphen (4-nitro-3,5-bisacetoxymercuri-2 cresol), formaldehyde, copper sulphate, tincture of iodine, chloramine-T (sodium toluene-p-sulphonchloramine), sodium hypochlorite, ethyl alcohol, sodium chloride, and hydrochloric acid.

The stock solutions of the chemicals studied were made with care. The solid compounds were weighed on accurate balances to within 0.1 mgm. of the theoretical amount; solutions were made in standard volumetric flasks; and calibrated pipettes were used in making the desired dilutions. Final concentrations of 10 per cent yeast and 1 per cent sugar were used throughout. The yeast suspensions and the sugar solutions were freshly made in distilled water each day, and were kept at 3 to 6°C. To set up the tests, equal parts of 20 per cent yeast and 2 per cent sugar, with the desired amount of test chemical were placed in a small flask, and the mixture distributed in 10-cc. amounts into the small tubes (c). Six tubes of each dilution of every chemical were routinely used in all experiments. These tubes, and also the control series referred to above, which contained only yeast and sugar, were all placed in a water bath at 38°C. The time elapsing between the mixing of the yeast, sugar, and chemical, and the immersion of the series of tubes in the water bath was approximately two minutes. Readings were made after fifteen minutes, thirty minutes, one hour, and every hour thereafter until fermentation ceased. Then curves were plotted with the readings obtained, each curve representing the average of 6 tubes containing the same material. It is impossible to present here charts which illustrate the action of all of the chemicals included in this study. Much of the information gleaned from the curves on these charts is presented in a condensed form in the accompanying table. (See table 1.)

The effect of mercuric chloride upon yeast fermentation is

TABLE 1

The effects of varying dilutions of sixteen chemical compounds upon the course of CO_2 production by yeast

EXPERIMENT NUMBER	CHEMICAL	DILUTION	AMOUNT CO₂ PRODUCED PER UNIT OF TIME					REMARKS
			Time in hours					
			1	2	3	4	5	
			cc.	cc.	cc.	cc.	cc.	
1	HgCl₂	5,000	0	0	0	0	0	Stimulation of fermentation
		10,000	2	6	8	9	10	during first 30 minutes in
		15,000	9	15	18	18	18	1:30,000 to 1:80,000
		20,000	13	19	19	19	19	Excess of total gas over that
		30,000	13	17	18	18	18	in control in 1:15,000 to
		50,000	13	14	15	15	15	1:80,000; greatest in
		80,000	13	14	14	14	14	1:20,000
		Control	14	14	14	14	14	
2	Mercuro-chrome	50	0	0	0	0	0	Excess of total gas over that
		80	3	4	4	4	4	in control in 1:1,000 to
		100	7	7	7	7	7	1:4,000
		200	9	10	11	12	13	
		500	10	10	11	11	11	
		1,000	10	13	16	16	16	
		2,000	11	14	17	17	17	
		4,000	14	15	16	16	16	
		Control	11	11	12	12	12	
3	AgNO₃	6,000	0	0	0	0	0	Practically no increase in
		8,000	1	2	3	5	8	fermentation over the
		10,000	3	10	11	11	11	control in any dilutions
		15,000	7	12	12	12	12	
		20,000	11	12	12	12	12	
		30,000	12	13	14	14	14	
		60,000	12	12	12	12	12	
		Control	12	12	12	12	12	
4	Phenol	100	0	0	0	0	0	Excess of gas over that in
		200	1	2	3	4	7	control in 1:250 to 1:600.
		250	3	8	12	17	21	Greatest total amount of
		300	4.5	10	15.5	16	18	gas in 1:350. Fermenta-
		350	6	16	17	18	21	tion much delayed in 1:200
		400	9	16	17	18	19	to 1:300
		600	11	15	15	15	15	
		Control	12	12	12	12	12	
5	Lysol	200	0	0	0	0	0	Excess of gas over that in
		300	0.5	1.0	2			control in 1:400 and 1:500.
		350	4.0	9.0	9.0			Greatest amount in 1:500

TABLE 1—*Continued*

EXPERIMENT NUMBER	CHEMICAL	DILUTION	AMOUNT CO_2 PRODUCED PER UNIT OF TIME					REMARKS
			Time in hours					
			1	2	3	4	5	
			cc.	cc.	cc.	cc.	cc.	
5	Lysol	400	8.0	16.0	18.0			.
		500	17.0	22.0	22.0			
		Control	15.0	16.0	16.0			
6	Tricresol	300	0	0	0	0	0	Late increase in fermentation in 1:500 to 1:1,000
		400	2	5				
		500	8	16				
		1,000	13	17				
		Control	11	13				
7	Hexylre- sorcinol	2,000	0	0	0	0	0	Increased fermentation in 1:5,000 to 1:10,000
		2,500	2	3	4	4	4	
		3,000	5	8	11			
		8,000	14	16	17	18		
		10,000	14	16	16	16		
		Control	12	12	12	12	12	
8	Metaphen	2,000	0	0	0	0	0	Increased fermentation in 1:5,000 to 1:10,000, greatest in 1:8,000, but quickest in 1:10,000
		3,000	0	0.5	1.0			
		3,500	1	4	4.5	5	6	
		4,000	2.5	9	10.0			
		5,000	5	13	15	15	16	
		6,000	8	16	17			
		8,000	11	20	21			
		10,000	15	18	18			
		Control	10	10	10	10	10	
9	Formal- dehyde	200	0	0	0	0	0	Very marked late increase in fermentation in 1:600 to 1:1,000
		400	1	3	5	6	7	
		600	3	12	16	20	20	
		800	2	9	19	20	21	
		1,000	11	19	19	19	19	
		Control	12	12	12	12	12	
10	Copper sulphate	80	2	2	2	2	2	Some increased fermentation in 1:10,000 to 1:20,000. Very slight, but definite fermentation, regardless of dilution, between 1:80 and 1:1,000
		1,000	2	2	2	2	2	
		2,000	3	4	4	4	4	
		6,000	4	6				
		8,000	8	12				
		10,000	11	13	15	16	16	
		20,000	12	13	14			
		Control	13	13	13	13	13	

TABLE 1—*Concluded*

EXPERIMENT NUMBER	CHEMICAL	DILUTION	AMOUNT CO_2 PRODUCED PER UNIT OF TIME					REMARKS
			Time in hours					
			1	2	3	4	5	
			cc.	cc.	cc.	cc.	cc.	
11	Tincture of iodine	250	0	0	0	0	0	Delayed fermentation in lower dilutions. Late increase in total gas formed in 1:500 to 1:1,000
		300	0	0	4	7	10	
		500	4	12	15	17	20	
		1,000	8	16	16	17	17	
		10,000	11	13	13	13	13	
		Control	13	14	14	14	14	
12	Chlora-mine-T	2,000	0	0	0	0	0	Vigorous fermentation after long latent period in 1:5,000
		5,000	1	7	16	22		
		7,000	6	10				
		8,000	8	12				
		10,000	9	11	12			
		20,000	13	13	14			
		Control	12	13	13			
13	Sodium hypo-chlorite*	50	0	0	0	1	1	Active fermentation after long latent period in lower dilutions
		75	0	3	6	8		
		100	0	5	9	9		
		200	9	10				
		500	10	11				
		1,000	11	11				
		5,000	13	14				
		Control	13	13	13	13	13	
14	Ethyl alcohol	(2%) 5	0	0	0	0	0	Slightly increased fermentation in 1:10
		(10%) 10	12	15	17			
		Control	12	13	13			
15	HCl	N/10	0	0	0	0	0	
		N/20	0	1	2	3	4	
		N/50	4	6	7	7	7	
		N/100	4	7	10	11	11	
		Control	6	7	9	11	12	
16	NaCl	5	0	0	0	0	0	Pronounced stimulation of fermentation by all dilutions higher than 1:10
		10	2	8	9	9	9	
		20	12	12	12	12	12	
		50	24	25	25	25	26	
		100	29	30	31	31	31	
		Control	9	9	9	9	9	

* The amount of free available chlorine in different samples of sodium hypochlorite varies widely, and the results obtained here would by no means be constant with other samples. With the sample represented in this table a dilution of 1:100 represented only 50 parts per million of free available chlorine.

shown in chart 1, which records observations made over a period of five hours. Carbon dioxide production was inhibited completely in dilutions up to 1:5000. Although a tremendous lag occurred in dilutions of 1:5000 to 1:10,000, an appreciable amount of gas was ultimately evolved. Fermentation was retarded in dilutions up to 1:80,000, but inhibition in some of these higher dilutions of mercuric chloride was only temporary for the total amount of carbon dioxide ultimately formed was often much greater than that in the control tubes containing only yeast
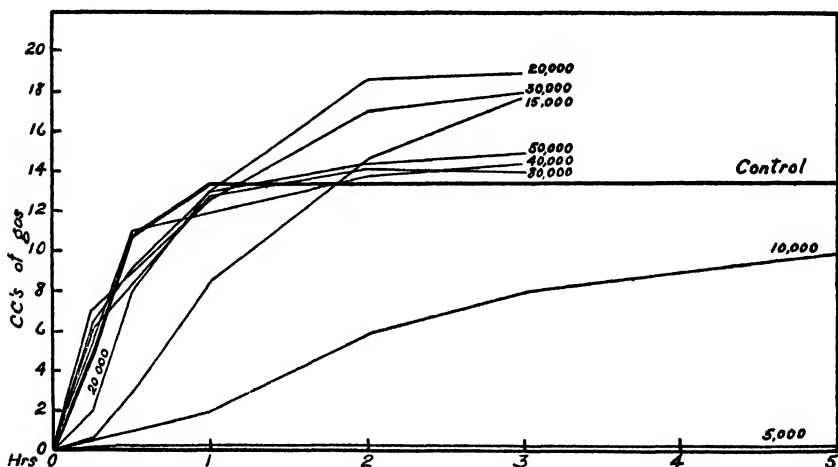
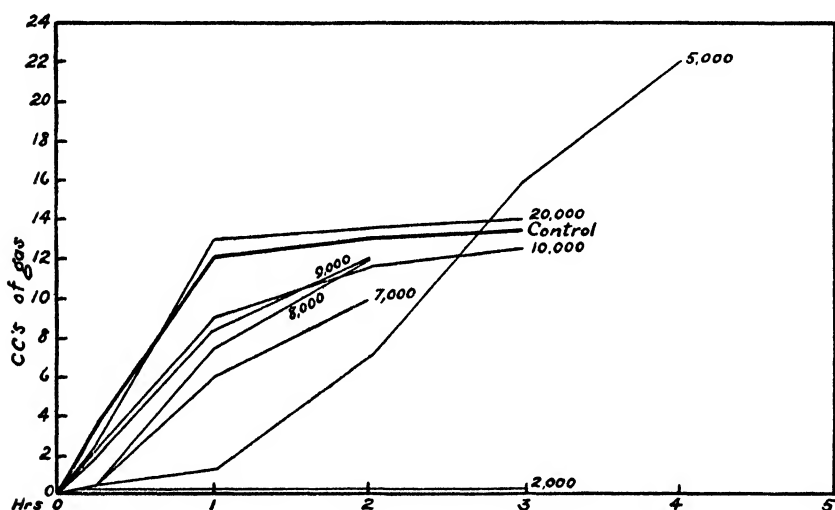CHART 1. EFFECT OF VARYING DILUTIONS OF MERCURIC CHLORIDE UPON CARBON DIOXIDE PRODUCTION BY 10 PER CENT YEAST AND 1 PER CENT SUCROSE

and sugar. It is evident from a study of these curves that this increase in the total gas, above the amount formed in the tubes without mercuric chloride, occurred after the normal reaction in the control series was practically finished. Readings made just at the completion of fermentation in the control tubes would show a definite inhibition by dilutions of mercuric chloride up to 1:80,000, the degree depending directly upon the concentration of the mercuric chloride. But if the total amount of gas produced, regardless of time, be taken as an end-point, readings might be interpreted to indicate stimulation of fermentative activity in certain of these same dilutions. Frequent readings

show the degree and duration of the inhibitory effect of different dilutions of mercuric chloride as well as the amount and rate of carbon dioxide production.   The maximum amount of gas was by no means always produced in tubes containing the minimum quantity of mercuric chloride which produced an effect.   Chart 1 shows that the greatest total carbon dioxide production was in dilutions of 1:15,000 to 1:30,000, the amount formed being 25 to 35 per cent greater than that produced in the tubes without mercuric chloride.   This excess of gas was evolved rather slowly, reaching the maximum at the end of about three hours, whereas fermentation in the tubes without mercuric chloride had ceased at the end of one hour.

Another effect of this compound may be seen in the curves representing dilutions of 1:30,000 to 1:80,000.   Here a genuine stimulation of fermentation seems to have occurred during the first fifteen minutes; after that the rate of carbon dioxide production fell off; at one hour the total amount was less than the control, and when fermentation was finally complete the excess was very little.   If the final observations had been made at some arbitrarily chosen time interval, the interpretation of the experiment would have differed greatly according to whether fifteen minutes, one hour, or three hours had been selected as the end-point.   With the simple apparatus described above it is possible to observe the whole process.

Thus we find, in the case of mercuric chloride, that low dilutions exert a marked inhibiting action upon fermentation; higher dilutions cause definite inhibition temporarily, but the total amount of carbon dioxide formed may be far in excess of that produced by the control tubes without mercuric chloride; and in some of the highest dilutions which effect the process at all there is an actual initial stimulation, followed by a decreased rate of fermentation.

The course of fermentation in the presence of mercuric chloride has been discussed here in detail because some of its phases are shown by other chemicals.   An excess of carbon dioxide above the amount in the control tubes was produced regularly in some dilutions of a number of compounds other than mercuric chloride,

when fermentation was allowed to proceed to completion. Almost invariably this phenomenon occurred after the cessation of fermentation in the control series of tubes, and usually in dilutions that caused a definite inhibition of fermentation during the first hour. Charts representing the action of phenol (see chart 2), lysol, tricresol, mercurochrome, formaldehyde, tincture of iodine, chloramine-T (see chart 3), copper sulphate, hexyl resorcinol, metaphen, ethyl alcohol, hydrochloric acid, and sodium chloride all indicate such an effect. Table 1 shows that in some of these cases the excess of gas produced beyond that in the
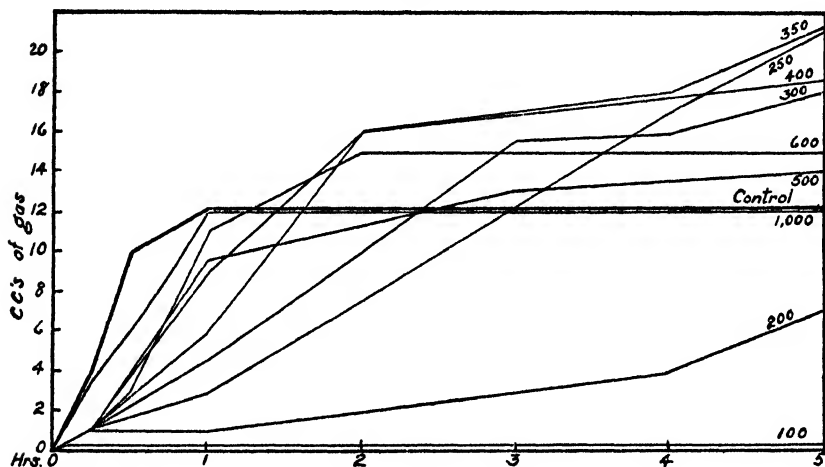
CHART 2 EFFECT OF VARYING DILUTIONS OF PHENOL UPON CARBON DIOXIDE PRODUCTON BY 10 PER CENT YEAST AND 1 PER CENT SUCROSE

controls was considerable; in formaldehyde there was 60 per cent more than the control in dilutions of 1:600 to 1:1000; in lysol, 25 per cent in 1:400 to 1:500; in phenol, 60 per cent in 1:350; in hexylresorcinol, 50 per cent in 1:8000; in sodium chloride, more than 200 per cent with a one per cent solution. With some other chemicals the excess was less. In most cases this excess gas was found in the highest dilutions of the chemicals that had caused any initial inhibition. But in others (phenol, chloramine-T, and tincture of iodine) the greatest final amount of gas was formed in concentrations that had caused marked inhibition for a consider-

able period of time. The most extreme case of that type was chloramine-T (chart 3) where tubes containing a concentration that completely prevented gas formation for one hour, ultimately, at the end of four hours, contained nearly twice as much carbon dioxide as the control, a ratio of 22 to 13 cc.

This increased fermentation in the presence of certain dilutions did not occur with all of the chemicals studied. None of the curves plotted for any sample of silver nitrate (chart 4) showed an appreciably increased amount of carbon dioxide beyond that

CHART 3. EFFECT OF VARYING DILUTIONS OF CHLORAMINE-T UPON CARBON DIOXIDE PRODUCTION BY 10 PER CENT YEAST AND 1 PER CENT SUCROSE

in the control tubes, nor did sodium hypochlorite show it during the period of observation.

Initial stimulation of fermentation by high dilutions, as was noted in the chart representing the action of mercuric chloride, can also be seen strikingly with sodium chloride. Here the stimulation is enormous, the total gas formed in 1 per cent sodium chloride being more than 3 times as much as in the controls (31 to 9 cc.) within the same period of time. The action of sodium chloride, and its effect upon the activity of other chemicals have been discussed by many. Pilcher and Sollmann (1923–1924) refer

to its action in preventing the inhibition of yeast fermentation by silver salts. Explanations of its action have been offered by Krönig and Paul (1896), by Rideal and Rideal (1921), by Halvorson and Cade (1928) and by Speakman, Gee and Luck (1928).

The action of copper sulphate was unique. With this salt there was some fermentation during the first hour, even in concentrations as great as 1:80, but only in dilutions greater than 1:5000 did any great degree of gas formation occur.

A similarity in the general behavior of yeast in the presence of related chemicals was apparent, even though the concentrations
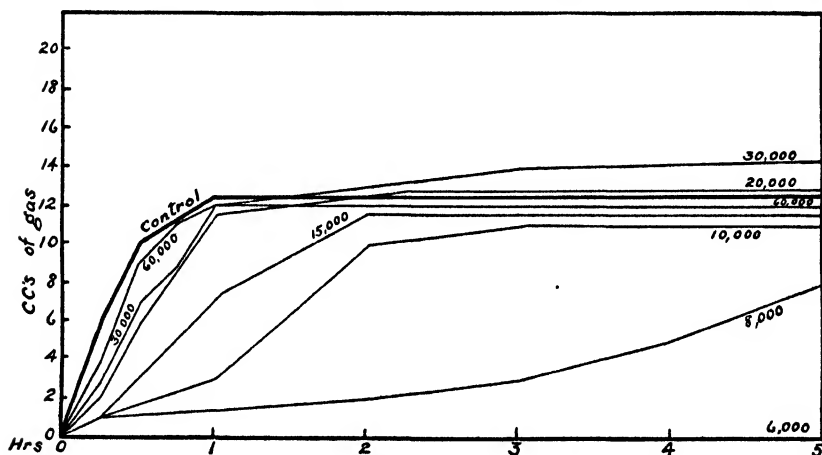
CHART 4. EFFECT OF VARYING DILUTIONS OF SILVER NITRATE UPON CARBON DIOXIDE PRODUCTION BY 10 PER CENT YEAST AND 1 PER CENT SUCROSE

necessary to produce the same effects varied widely in individual members of these groups. For example, the curves representing the effect of mercurochrome definitely resemble those of mercuric chloride, showing the same phases, although a concentration of 1:50 of mercurochrome is necessary to produce the same degree of inhibition of fermentation that is caused by 1:5000 mercuric chloride. The charts showing the effect of phenol (chart 2), lysol, and tricresol are very similar to each other in general appearance, as are also those for two creosote oil preparations examined. Within a wide range of dilutions (see chart 2) the

curves are the same for the first 15 minutes, after which they become very divergent. Charts representing the curves plotted for those compounds which liberate free halogen; viz., sodium hypochlorite, chloramine-T (chart 3), and tincture of iodine, show an especially striking resemblance to each other. The most remarkable feature of these is a sudden abundant gas formation in concentrations that completely inhibited fermentation for one or two hours.

SUMMARY

A simple gasometer has been devised in which carbon dioxide production by yeasts can be measured with a fair degree of accuracy, and the course of the fermentation observed. This apparatus offers a means of studying some interesting phases of the process of fermentation under the influence of many agents.

The concentration at which fermentation is completely inhibited has been readily determined for every chemical studied. An apparent stimulation of carbon dioxide production by high dilutions of compounds that inhibited this activity in greater concentrations often occurred, the total amount of gas produced frequently being far in excess of that evolved by an equal amount of the yeast-sugar mixture to which no chemical had been added.

Curves plotted with readings made at frequent intervals show that this increased fermentation commonly occurred in dilutions that had definitely inhibited activity for a greater or less period of time. This phenomenon was manifested by the majority of compounds studied, though their behavior was by no means identical. Sometimes the greatest amount of gas was formed in dilutions which caused relatively slight initial inhibition; i.e., mercuric chloride, mercurochrome, metaphen, and hexylresorcinol. With other chemicals a sudden outburst of activity occurred in dilutions which had caused complete inhibition for one or two hours; i.e., those which liberated free halogen such as chloramine-T, tincture of iodine, and sodium hypochlorite.

In some instances there was a transient initial stimulation in dilutions too high to exert any inhibitory action; i.e., mercuric chloride and sodium chloride.

A few compounds showed no phases of increased fermentation in any dilutions; i.e., silver nitrate.

The data presented indicate that the effect of individual chemicals is characteristic, and that related compounds act upon the yeasts in a similar way.

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THE INFLUENCE UPON BACTERIAL VIABILITY OF VARIOUS ANIONS IN COMBINATION WITH SODIUM[1]

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I. INTRODUCTION

A vast number of studies have been made on the effect of cations upon the course of various vital phenomena. Loeb (1912) reviewed the earlier literature of the subject (largely dealing with animal cells and tissues); while Falk (1923) has prepared a more recent summary, particularly with reference to the effects of salts upon bacterial viability. The net effect of the studies conducted with animal cells and tissues of higher plants has been to indicate that, in the concentrations commonly used, the univalent cations are generally favorable to vital activity while the bivalent cations are unfavorable. In general, it has been indicated that the monovalent ions increase permeability while the bivalent ions decrease it. In mixtures of monovalent and bivalent salts "antagonism" effects appear, both in regard to viability and permeability. (See McCutcheon and Lucke, 1928, for a recent contribution along this line.)

Investigations upon bacterial cells, largely conducted in this laboratory (Hotchkiss, 1923; Winslow and Falk, 1923; Falk and Winslow, 1926; Winslow and Dolloff, 1928) have suggested a somewhat different interpretation of these phenomena. Their results indicate that it is erroneous to consider a given cation as tending to favor or inhibit bacterial growth, to increase or

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decrease permeability. They suggest rather that the whole problem is a quantitative one. All cations appear to stimulate bacterial growth (and probably to increase permeability) at a low concentration and to produce an opposite effect in a higher concentration. The supposed specific effects are due merely to the fact that different cations have different quantitative potencies; and antagonistic or additive effects will appear according to the quantitative relationships involved.

Studies of anion effects have been less numerous but a considerable literature has grown up around the effect of the hydroxyl ion. Among the more outstanding studies which have dealt with this phase of the subject may be mentioned those of Cohen and Clark (1919), Falk (1920), Cohen (1922), Winslow and Shaughnessy (1924), Shaughnessy and Falk (1924), Shaughnessy, and Winslow (1927) and Myers (1928). These investigations have made it clear that very slight increases or decreases in hydrogen ion concentration at the limits of the optimum zone produce marked effects on bacterial viability, effects which the bacterial cells tend to neutralize by the liberation of acidic or basic substances, respectively.

Kligler (1918) and Graham-Smith (1919) studied the effect of hydrogen ion concentration upon the antiseptic effect of various dyes, with various results. Davis and White (1918), Browning, Gulbransen and Kennaway (1919) and Davis (1921a and 1921b) reported that acriflavine and proflavine, acridine compounds and certain anilin dyes were more toxic in alkaline solution. Burke and Grieve (1924) obtained similar results. Stearn and Stearn (1924a, 1924b and 1924c) advanced a general theory to explain the bacteriostatic action of dyes on the assumption that bacterial protoplasm acts as an amphoteric colloid, generally having a greater affinity for basic dyes. Tilley and Schaffer (1925) found that an alkaline reaction increased the antiseptic power of soaps while Halvorsan and Cade (1928) reported that phenol derivatives were more effective in an acid medium.

The special problem with which the present investigation is concerned,—the simultaneous effect of cations and hydrogen ion concentration upon bacterial viability,—was first investigated

in detail by Sherman and Holm (1921 and 1922) and Winslow and Falk (1922). Sherman and Holm find that sodium salts broaden the optimum zone of hydrogen ion tolerance and Winslow and Falk find that calcium salts narrow it. They explain this phenomenon in the case of calcium as due to interference by the calcium with the diffusion from the cell of neutralizing acidic substances (decreased permeability effect). Shaughnessy and Winslow (1927) confirmed this theory by direct chemical tests.

A particularly important contribution to this subject has recently been made by Levine and his colleagues (Levine, Buchanan and Lease, 1927; Levine, Buchanan and Toulouse, 1927; Peterson, Levine and Buchanan, 1927; Levine, Peterson and Buchanan, 1927; Levine, Peterson and Buchanan, 1928; Levine, Toulouse and Buchanan, 1928). The net result of their work is to indicate that the toxic effect of sodium hydroxid upon the spores of bacteria (subtilis group) and yeasts is markedly increased by the presence of salts ($NaCl$, Na_2CO_3 and Na_3PO_4). The effect is too great to be explained by any merely additive effect of the cation and the alkalinity and the authors attribute it to an increase in concentration of undissociated sodium hydroxid or to a decreased solubility of sodium hydroxid in the water phase due to the presence of the salt. They believe the undissociated sodium hydroxid is probably the actual toxic agent.

The problem with which we are dealing in such a case as this is obviously a highly complex one. Addition of a salt to an alkali will, as Levine and his co-workers suggest, produce, first of all, direct chemical effects on dissociation and solubility. Secondly, as Winslow and Falk have pointed out, the salt may effect the permeability of the cell wall and thus influence the rate of penetration of alkali into the cell and of neutralizing substances out from the cell. Thirdly, there is always to be considered the additive or antagonistic effect of the added ions or molecules upon the bacterial protoplasm. Furthermore, the effects with spores may be quite different from those which are manifest with vegetative cells, on account of the different relative part presumably played by permeability and toxicity phenomena in the two instances.

The object of the present study has been to throw some further light on the problem as it manifests itself in the case of vegetative cells, using sodium as a common cation in a combination with hydroxyl and other anions which would yield a varying range of hydrogen ion concentrations.

II. EXPERIMENTAL TECHNIQUE

The organism used in these experiments was the same strain of *Escherichia coli* (*communis* type) employed by Winslow, Falk, Cohen, Hotchkiss, Shaughnessy and Dolloff in previous studies made in this laboratory. It was maintained on standard nutrient agar. All glassware employed was of Pyrex or equally insoluble type, cleaned by soaking in cleaning solution for twenty-four hours, rinsed in tap water and then in distilled water and sterilized in dry heat at 160°C. for five hours. The distilled water used was prepared in a Barnstead still. It gave negative tests for ammonia and chlorides and had a hydrogen ion concentration between pH 5.9 and pH 6.6.

The actual viability tests were made in the synthetic medium described by Dolloff (1926) (containing 5 grams of ammonium tartrate, 5 grams of lactose and 0.02 gram of dibasic ammonium phosphate to the liter), and in the one per cent Bactopepton medium used by Hotchkiss (1923). The ammonium tartrate was recrystallized and the lactose was a special c.p. Pfanstiehl product, represented as free from sulphates, chlorides, aluminum, calcium and heavy metals. This medium was sterilized in the autoclave for twenty minutes at a pressure just under 15 pounds and had a pH after sterilization of 5.4. The Bactopepton was a Digestive Ferments Company product.

The substances, whose effects were tested in these fundamental media were $NaCl$, $NaHCO_3$, Na_2CO_3, $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 12 H_2O$, $Na_3PO_4 \cdot 12 H_2O$, Na_2SO_4 and $NaOH$. All were Baker's analyzed products, of tested purity. In making up the salt solutions, the salts were weighed out on clean sterile glasses and placed in the desired amount of sterile distilled water. The solutions were then allowed to stand for 24 hours and tested for sterility. This procedure was adopted in view of the effect of

sterilization upon certain salts, particularly the bicarbonate. The hydroxid solutions were made of a higher strength than those desired and weaker solutions prepared by dilution. The final test solution in which viability was to be studied was prepared by mixing appropriate amounts of salt solution, or hydroxid solution, or both, with the Dolloff or pepton medium so as to give the ultimate molal concentration desired.

In making an experiment, *Escherichia coli* was transferred from standard agar to Dolloff's medium and grown for twenty-four hours at 37°C. One cubic centimeter of the Dolloff medium culture was then added to the test solution,—Dolloff medium containing the desired molal concentration of chemicals. Each cubic centimeter of this test solution as thus inoculated contained about 10 million bacteria. The test solution was then incubated for eighteen to twenty hours at 37°C. and the number of bacteria present then determined by plating on standard nutrient agar. Plates were made in duplicate or triplicate.

Hydrogen ion determinations were made by both the colorimetric and electrometric methods. The indicators and standard solutions used in the colorimetric method were prepared by the LaMotte Chemical Products Company (LaMotte purple being used for the higher alkaline range). Electrometric measurements were made either with a Youden hydrogen-ion apparatus or with a Leeds and Northrup students' potentiometer. All determinations at pH values above 8.2 were made colorimetrically. Due corrections were made for salt effects.

III. RESULTS OF A TYPICAL SERIES OF EXPERIMENTS

In the course of the work here reported, the viability of the test organism was determined in Dolloff medium with 15 different salt mixtures by the technique described above, while specially washed cells were tested with three different salt mixtures in Dolloff's medium and with three in pepton medium. For each of these 21 combinations, between 7 and 18 different salt concentrations were studied. There were 218 different viability points thus determined and each point was checked by from 4 to 9 separate determinations, giving a total of 1422 different tests. Approximately 3700 plates were made in the entire series.

TABLE 1

Viability of Escherichia coli in the presence of various concentrations of NaCl,
NaOH and NaOH plus NaCl in Dolloff's medium

MOLALITY	NUMBER OF EXPERIMENTS	pH	MILLIONS BACTERIA PER CUBIC CENTIMETER	PER CENT EXCESS
NaCl				
0	6	5 4	65	
0 001	6	5.7	80	+22
0 005	6	5 7	104	+59
0 01	6	5 7	99	+52
0 025	6	5.7	118	+81
0.05	6	5 7	136	+109
0 1	6	5 7	155	+138
0 20	6	5 7	127	+94
0 25	6	5.7	114	+90
0 3	6	5.7	89	+36
0.4	6	5 7	65	0
0 5	6	5 7	43	−33
0 6	6	5 7	18	−72
0.7	6	5 7	3	−95
0 8	6	5.7	1	−98
1	6	5 7	0.28	−99
NaOH				
0	7	5 4	107	
0 0005	7	5 9	111	+4
0.001	7	7.0	116	+8
0 002	7	7.6	123	+15
0.004	7	8 0	146	+36
0 006	7	8 4	142	+32
0 008	7	8 4	96	−10
0 01	7	8 5	3	−98
0 02	7	8.8	0.13	−99
NaOH + NaCl				
0	9	5 4	101	
0 0001	9	5 8	109	+7
0.0005	9	5.8	104	+2
0 001	9	6 6	113	+11
0 002	9	7.0	130	+29
0 004	9	7.6	148	+46
0 006	9	7.8	168	+59
0.008	9	8.0	178	+75
0 01	9	8.3	135	+33
0.02	9	8.4	17	−84
0.03	9	8 6	0.45	−99

Table 1 will serve to illustrate the type of results obtained. In the first column are indicated the molal concentrations of sodium in each of the test solutions (irrespective of whether the sodium was combined with chlorine or hydroxid). In the second column are the number of independent tests averaged to give the indicated results,—6 in the case of each concentration of NaCl, 7 in the case of each concentration of NaOH and 9 in the case of each concentration of the mixture. In the third column is given the pH of the medium, in the fourth the number of millions of bacteria per cubic centimeter after eighteen to twenty hours' incubation at 37°C. In the last column is the excess of the final count at a given salt concentration, expressed as a percentage of the final count in the Dolloff medium with no added salt.

It has been pointed out above that the number of bacteria originally inoculated amounted to about 10 million per cubic centimeter. After incubation, as table 1 shows, the number amounted to 65 million in the NaCl series control, to 107 million in the NaOH control and to 101 million in the control run for the mixture. In the 15 series of tests carried out in Dolloff's medium by the procedure outlined above, the average control counts in Dolloff medium alone for each series varied from 65 million to 178 million and averaged 133 million. The average control for each series has been taken as the base line for that series since it was run at the same time and under the same conditions.

The results in table 1 are presented graphically in figure 1. The abscissae represent logs of Na concentration, the ordinates per cent excess of final counts as compared with final counts in the control containing no salts. It will be noted that with either NaCl, NaOH or a mixture of the two, a low concentration of sodium has a stimulating effect, as compared with the Dolloff medium alone, while a further increase of sodium concentration produces an inhibiting effect. Both stimulating and inhibiting effect are manifest with much lower concentrations of sodium in the form of NaOH than in the form of NaCl, while the mixture occupies an intermediate position. The main contention of Winslow and Dolloff (1928) that the difference between cations in their effect on bacterial viability is a quantitative one is sustained and extended to include NaOH.
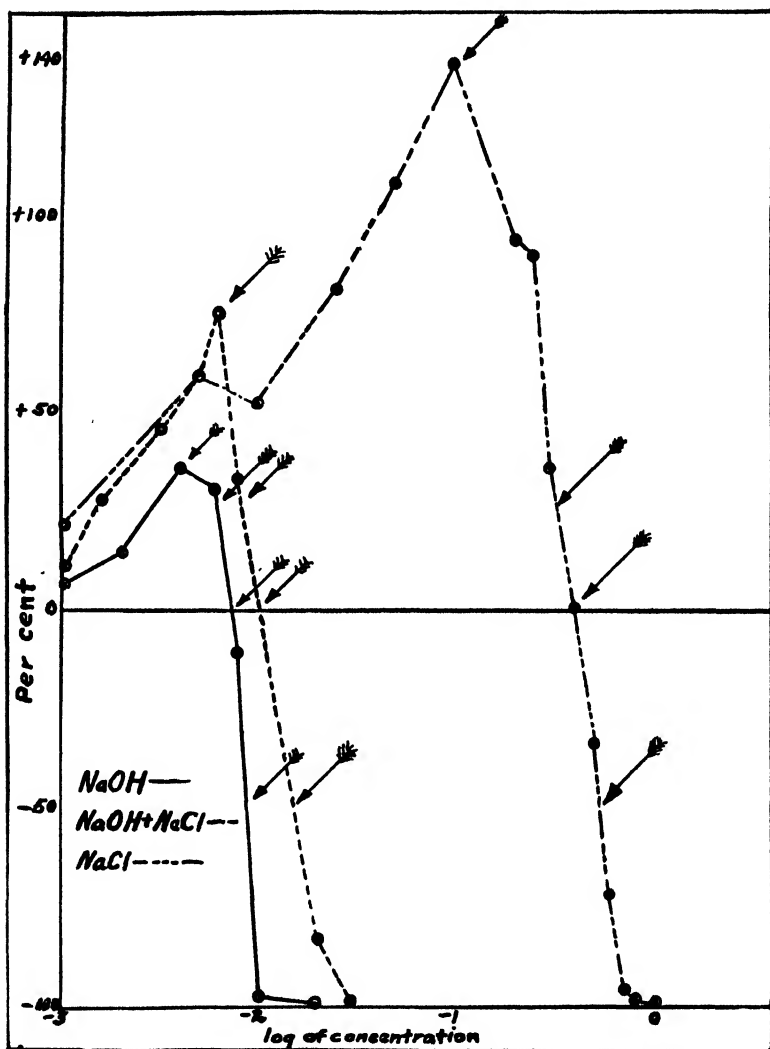
Fig. 1. Viability Curve at Different Concentrations of NaOH, NaCl and NaOH + NaCl in Dolloff's Medium

Throughout all our tests exactly the same phenomenon is manifest. Every salt solution and every mixture of salt and sodium hydroxid showed a curve of the same general form and

in every case but one, the mixture of salt and hydroxid gave a curve intermediate between that for the salt concerned and that for the hydroxid alone.   This exception occurred with NaH_2PO_4 and will be discussed later.

TABLE 2

Viability of Escherichia coli in the presence of various concentrations of $NaHCO_3$, NaOH and NaOH + $NaHCO_3$

MOLALITY	NUMBER OF EXPERIMENTS	pH	MILLIONS BACTERIA PER CUBIC CENTIMETER	PER CENT EXCESS
NaHCO₃				
0	7	5.4	152	
0.0005	7	6.3	176	+16
0 001	7	6.7	176	+16
0.005	7	7.3	183	+20
0.01	7	7.4	199	+31
0 02	7	7.5	196	+29
0.04	7	7.6	251	+65
0 06	7	7.9	294	+92
0 08	7	8.0	221	+45
0.1	7	8 0	166	+9
0.2	7	8 0	68	−55
0 3	7	8.1	2	−99
0 4	7	8.1	0 57	−99
0.5	7	8.1	0.1	−99
NaOH + NaHCO₃				
0	9	5.4	168	
0.001	9	6 0	157	−7
0.002	9	6.4	181	+8
0 004	9	7 0	206	+23
0.006	9	7.6	259	+54
0 008	9	7.7	280	+67
0.01	9	7.8	235	+40
0 02	9	7.9	63	−63
0.03	9	8.4	35	−79
0.04	9	8.5	3	−98

In view of the general uniformity of results it has been possible for comparative purposes to select certain outstanding points on the curves; and for this purpose, four points have been arbitrarily selected which give a more concise representation of the curve as a whole.   Since each curve shows, with increasing concentration

of sodium, first an increase in relative numbers of bacteria up to a maximum followed by a decrease to a figure below the control the points chosen are as follows:

 a. The concentration of sodium giving a 25 per cent excess over the Dolloff control.

TABLE 3

Viability of Escherichia coli in the presence of various concentrations of Na_2CO_3 and Na_2CO_3 + NaOH

MOLALITY	NUMBER OF EXPERIMENTS	pH	MILLIONS BACTERIA PER CUBIC CENTIMETER	PER CENT EXCESS
Na_2CO_3				
0	7	5 4	140	
0.0001	7	6 2	134	−0 04
0.0005	7	6 2	137	−0.02
0 001	7	7 2	146	+0 04
0 002	7	7 6	174	+24
0 004	7	8.0	220	+57
0 006	7	8 2	265	+89
0 008	7	8 2	270	+93
0.01	7	8 6	125	−0 17
0 02	7	9 0	2	−98
0 03	7	9 4	0.13	−99
NaOH + Na_2CO_3				
	6	5.4	155	
0.0001	6	5 8	144	−0.07
0 0005	6	6 4	178	+15
0.001	6	7 0	184	+19
0 002	6	7 6	222	+43
0.004	6	7.8	265	+71
0 006	6	8 0	238	+53
0.008	6	8.3	146	−0 54
0 01	6	8 6	14	−92
0 02	6	9.2	1	−99

 b. The concentration giving a maximum count.

 c. The concentration (on the descending arm of the curve) giving a count identical with the control.

 d. The concentration giving a 50 per cent reduction in numbers, as compared with the control.

TABLE 4

Viability of Escherichia coli in the presence of various concentrations of NaH_2PO_4 and $NaOH + NaH_2PO_4$

MOLALITY	NUMBER OF EXPERIMENTS	pH	MILLIONS BACTERIA PER CUBIC CENTIMETER	PER CENT EXCESS
		NaH_2PO_4		
0	5	5.4	178	
0 005	5	5.5	158	+11
0.01	5	5.4	180	+14
0 02	5	5 3	199	+26
0.04	5	5 2	242	+53
0 06	5	5 0	236	+49
0 08	5	4.9	224	+42
0 1	5	4 9	162	+3
0.2	5	4.6	35	−77
0 3	5	4 4	9	−94
0 4	5	4 2	1	−98
0 5	5	4 1	0 1	−99
		$NaOH + NaH_2PO_4$		
0	6	5 4	166	
0 004	6	6 7	173	+0 41
0 006	6	7 1	187	+12
0 008	6	7.3	224	+35
0 01	6	7 4	257	+55
0.02	6	7.6	354	+113
0.04	6	7 5	535	+222
0.06	6	7 5	865	+420
0 08	6	7 5	1545	+832
0.1	6	7.8	1285	+673
0.2	6	7.9	1018	+512
0 3	6	7.9	829	+399
0 4	6	7.9	947	+470
0 5	6	7.9	720	+333
0.6	6	7.7	711	+328
0.7	6	7.7	107	−42
0.8	6	7.7	82	−51
1	6	7.7	11	−93

It will be noted from table 1 and figure 1 that with NaCl, the 25 per cent excess is shown at a molal sodium concentration of 0.001, the maximum at 0.100; the cross-over point (count on descending arm equal to control) at 0.400 and the 50 per cent

decrease at 0.550. These points are indicated by arrows in figure 1.

For NaOH, the 25 per cent increase is at a molal sodium concentration of 0.003, the maximum at 0.004, the cross-over at 0.007 and the 50 per cent decrease at 0.009.

TABLE 5

Viability of Escherichia coli in the presence of various concentrations of Na_2HPO_4 and $NaOH + Na_2HPO_4$

MOLALITY	NUMBER OF EXPERIMENTS	pH	MILLIONS BACTERIA PER CUBIC CENTIMETER	PER CENT EXCESS
Na_2HPO_4				
0	4	5.4	130	
0.001	4	6.8	134	+3
0.005	4	7.4	187	+44
0.01	4	7.5	215	+65
0.02	4	7.8	250	+92
0.04	4	8.0	349	+196
0.06	4	8.1	353	+172
0.08	4	8.2	323	+148
0.1	4	8.3	274	+111
0.2	4	8.4	121	−7
0.3	4	8.4	0.02	−99
$NaOH + Na_2HPO_4$				
0	4	5.4	130	
0.001	4	7.2	128	−1
0.002	4	7.5	150	+16
0.004	4	8.0	178	+37
0.005	4	8.0	204	+57
0.006	4	8.0	235	+81
0.008	4	8.2	279	+115
0.01	4	8.3	284	+119
0.02	4	8.5	169	+30
0.04	4	8.8	0.59	−99

For the mixture of NaCl and NaOH in equal molal proportion; the 25 per cent increase is at a total molal sodium concentration of 0.002, the maximum at 0.008, the cross-over at 0.014 and the 50 per cent decrease at 0.018.

Further analysis of our results will be presented on the basis of

a comparison of these four significant points in the curve for each solution studied; but the average figures for each salt and salt mixture are presented for reference in tables 2 to 7.

TABLE 6

Viability of Escherichia coli in the presence of various concentrations of Na_3PO_4 and $NaOH + Na_3PO_4$

MOLALITY	NUMBER OF EXPERIMENTS	pH	MILLIONS BACTERIA PER CUBIC CENTIMETER	PER CENT EXCESS
Na_3PO_4				
0	7	5.4	150	
0.0001	7	5.8	131	−13
0.0005	7	6.6	142	−5
0.001	7	7.0	158	+6
0.002	7	7.5	169	+13
0.004	7	7.8	203	+35
0.006	7	8.2	246	+64
0.008	7	8.4	275	+84
0.01	7	8.6	125	−17
0.02	7	9.2	3	−98
$NaOH + Na_3PO_4$				
0	5	5.4	160	
0.0001	5	6.0	148	−9
0.0005	5	6.6	152	−5
0.001	5	7.4	172	+8
0.002	5	7.8	182	+8
0.004	5	8.2	253	+56
0.006	5	8.4	285	+74
0.008	5	8.6	210	+31
0.01	5	8.8	17	−89
0.02	5	9.2	0.24	−99

IV. INFLUENCE OF THE BASIC MEDIUM UPON VIABILITY

Before proceeding to an analysis of the influence of the various anions studied it will be desirable to summarize a few experiments in which the basic mode of procedure was modified, since these experiments indicate the essential importance of uniform technique, if comparable results are to be obtained.

The fundamental procedure followed involved, as pointed out above, the cultivation of *Esch. coli* in Dolloff medium and direct transfer from a twenty-four hour culture into a Dolloff medium test solution containing the salts studied.   From six to eight

TABLE 7

Viability of Escherichia coli in the presence of various concentrations of Na_2SO_4 and NaOH + Na_2SO_4 in Dolloff's medium

MOLALITY	NUMBER OF EXPERIMENTS	pH	MILLIONS BACTERIA PER CUBIC CENTIMETER	PER CENT EXCESS
Na_2SO_4
0	8	5.4	88	
0.0005	8	5.5	83	−3
0.001	8	5.5	105	+19
0.005	8	5.5	110	+25
0.01	8	5.5	130	+48
0.05	8	5.5	149	+69
0.08	8	5.5	147	+67
0.10	8	5.5	108	+23
0.25	8	5.5	84	−4
0.5	8	5.5	45	−49
NaOH + Na_2SO_4
0	8	5.4	100	
0.00005	8	5.5	117	+17
0.0001	8	5.5	116	+16
0.0005	8	5.7	126	+26
0.001	8	5.7	152	+52
0.005	8	5.8	180	+80
0.008	8	7.5	152	+42
0.01	8	7.8	123	+23
0.02	3	8.1	52	−51
0.03	3	8.4	9	−91
0.04	3	8.6	0.013	−99
0.05	8	8.8		

different series of tests were made with NaCl, NaOH and a mixture of the two, in which the organism was cultivated for twenty-four hours on standard nutrient agar, washed off with sterile distilled water, and washed and centrifuged three times in

sterile distilled water.   These washed cells were then inoculated into the Dolloff medium test solution.   This procedure had the effect of removing any protective metabolic products which might be present in the zones immediately surrounding the cells.   The pH of the wash water was slightly increased by contact with the cells.

TABLE 8

Viability of Escherichia coli as affected by previous washing of cells, and by composition of basic medium

(1)	(2)	(3)		(4)		(5)	
		DOLLOFF MEDIUM UNWASHED CELLS		DOLLOHF MEDIUM WASHED CELLS		PEPTON MEDIUM WASHED CELLS	
SIGNIFICANT POINT	ADDED SUBSTANCE	(a) Effective molal concentration of Na	(b) pH	(a) Effective molal concentration of Na	(b) pH	(a) Effective molal concentration of Na	(b) pH
25 per cent increase	NaCl	0.001	5.7	0.0100	6.0	0.005	6.9
	NaOH	0.003	7.8	0.0007	6.1	0.001	7.3
	Mixture	0.002	7.0	0.0003	5.8	0.005	8.3
Maximum	NaCl	0.100	5.7	0.080	6.0	0.080	6.9
	NaOH	0.004	8.0	0.001	6.3	0.001	7.3
	Mixture	0.008	8.0	0.005	8.1	0.005	8.3
Cross-over	NaCl	0.400	5.7	0.270	6.0	0.410	6.9
	NaOH	0.007	8.4	0.005	8.1	0.005	8.3
	Mixture	0.014	8.3	0.007	8.2	0.007	8.5
50 per cent decrease	NaCl	0.550	5.7	0.400	6.0	0.500	6.9
	NaOH	0.009	8.4	0.007	8.3	0.008	8.6
	Mixture	0.018	8.4	0.009	8.4	0.009	8.9

All NaCl results based on average of 6 experiments; NaOH results on 7 to 8 experiments, results for mixture on 7 to 9 experiments.

Another series of 6 to 8 experiments was conducted with the same three electrolytes using washed cells, prepared as outlined above, but employing the 1 per cent pepton medium of Hotchkiss as a basic test solution instead of the Dolloff medium.

The results of these two series of experiments are summarized, —for the four significant points only,—in table 8.   The columns 3a and 3b of the table include data from table 1 for comparison.

Comparison of columns 3a and 4a of the table indicates that in every instance but one (NaCl, 25 per cent increase) the effective concentration for either stimulation or inhibition is much lower in the case of the washed cells than in the case of the unwashed cells. The washed cells, in other words, are more sensitive to their environment.

Comparing columns 4a and 5a, it appears that, when washed cells are used, the toxic effect of the salts is about the same in either Dolloff medium or pepton solution. This is indicated by the essentially concordant results in these two columns for the maximum point, the cross-over and the 50 per cent reduction. The maximum point is, of course, really determined by an inhibiting effect since stimulation goes on until inhibition begins. On the other hand, the pure stimulating effect, as measured by the point of 25 per cent increase is lower with NaCl and higher with the other two solutions in pepton than in Dolloff medium. Whether this difference is significant seems uncertain. The point of 25 per cent increase is always more variable than the other points studied.

V. SUMMARY OF RESULTS IN RESPECT TO THE INFLUENCE OF THE VARIOUS ANIONS STUDIED

We return now to our major problem,—the influence of the various anions studied upon the viability of *Esch. coli* in Dolloff's medium.

The results for the four significant points for each salt and salt mixture are presented in table 9. It should be understood that every combination studied showed essentially the same type of curve presented in figure 1 so that these four points are really indicative of the curve as a whole. The concentrations presented in columns 3a, 4a, 5a and 6a of table 9 represent in the case of the monovalent salts the actual molal concentration producing a given effect. In the case of bivalent compounds, however, the actual molal concentration has been multiplied by 2 and in the case of trivalent compounds by 3, so as to give a measure of the effect of each mixture on the basis of an equal Na concentration. This procedure was followed on the assumption (indicated by the work

of Winslow and Dolloff and others in this laboratory) that it is the Na ion which is significant in cation effects.   Thus, when it is stated in table 9 that a 25 per cent increase occurred in a 0.004 M solution of Na, it should be understood that the actual molal concentration of Na_2CO_3 was 0.002 M.   In the case of a mixture of a bivalent salt with NaOH, the actual molal concentration of combined salts was multiplied by 1.5.

TABLE 9

The molal concentrations of different sodium compounds corresponding to four different points on the viability curve calculated on the basis of the amount of sodium present in the solution

(1)	(2)	(3)		(4)		(5)		(6)	
		(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
ADDED SUBSTANCE	NUMBER TESTS	25 per cent increase	pH	Maximum point stimulation	pH	Cross over point	pH	50 per cent decrease	pH
NaOH	7	0.003	7.6	0.004	8.0	0.007	8.4	0.009	8.5
Na₂CO₃	7	0.004	7.6	0.016	8.2	0.020	8.6	0.030	8.8
NaOH + Na₂CO₃	6	0.002	7.0	0.006	7.8	0.012	8.3	0.018	8.4
Na₃PO₄	7	0.009	7.6	0.024	8.4	0.027	8.5	0.045	8.8
NaOH + Na₃PO₄	5	0.006	7.8	0.012	8.4	0.017	8.7	0.018	9.0
NaHCO₃	7	0.008	7.3	0.060	7.9	0.120	8.0	0.200	8.0
NaOH + NaHCO₃	9	0.004	7.0	0.008	7.7	0.014	7.8	0.018	7.8
Na₂HPO₄	4	0.006	7.3	0.080	8.1	0.380	8.3	0.500	8.4
NaOH + Na₂HPO₄	4	0.004	7.7	0.015	8.3	0.033	8.5	0.045	8.6
NaCl	6	0.001	5.7	0.100	5.7	0.400	5.7	0.550	5.7
NaOH + NaCl	9	0.002	7.0	0.008	8.0	0.014	8.3	0.018	8.4
Na₂SO₄	8	0.010	5.5	0.100	5.5	0.400	5.5	1.000	5.5
NaOH + Na₂SO₄	8	0.001	5.7	0.0075	6.8	0.015	7.8	0.030	8.1
NaH₂PO₄	5	0.020	5.3	0.0400	5.2	0.100	4.8	0.180	4.5
NaOH + NaH₂PO₄	6	0.007	7.2	0.0800	7.5	0.680	7.7	0.800	7.7

It will be noted from inspection of table 9 that the concentration giving the point of 25 per cent increase does not vary very widely.   The smallest molal concentration showing this degree of stimulation was 0.001 M NaCl and the highest, 0.020 NaH_2PO_4. Of the 15 figures in column 3a, 8 show a molal concentration between 0.001 and 0.004 and only 2 exceed 0.009.

The inhibiting concentrations, as indicated in columns 4a, 5a

and 6a differ much more widely, with NaOH always most potent, followed by Na_2CO_3 and Na_3PO_4, as indicated by the lower concentrations yielding a given effect; and with NaCl, Na_2SO_4 and the mixture of NaOH + NaH_2PO_4 always least potent.

The mixtures of NaOH with the various salt solutions almost invariably exhibit an effect intermediate between those of the NaOH alone and the particular salt alone. Taking all the salts

TABLE 10

Effective concentrations of various salts and salt mixtures computed on the basis of equal Na-concentration and referred to corresponding effective concentrations of NaOH as unity

(1)	(2)		(3)		(4)		(5)	
	25 per cent increase		Maximum		Cross-over		50 per cent decrease	
COMPOUND	(a) Conc.	(b) pH	(a) Conc.	(b) pH	(a) Conc	(b) pH	(a) Conc.	(b) pH
NaOH	1	7.6	1	8.0	1	8.4	1	8.5
Na_2CO_3	1	7.6	4	8.2	2	8.6	3	8.8
Na_3PO_4	3	7.6	6	8.4	4	8.5	5	8.8
NaH_2PO_4	6	5.3	10	5.2	14	4.8	20	4.5
$NaHCO_3$	3	7.3	15	7.9	17	8.0	22	8.0
Na_2HPO_4	2	7.3	20	8.1	54	8.4	55	8.4
NaCl	0.3	5.7	25	5.7	57	5.7	61	5.7
Na_2SO_4	3	5.5	25	5.5	57	5.5	111	5.5
NaOH + Na_2CO_3	0.6	7.0	1	7.8	2	8.3	2	8.4
NaOH + Na_3PO_4	2	7.8	3	8.4	2	8.7	2	9.0
NaOH + NaH_2PO_4	2	7.2	20	7.5	97	7.7	89	7.7
NaOH + $NaHCO_3$	1	7.0	2	7.7	2	7.8	2	7.8
NaOH + Na_2HPO_4	1	7.7	4	8.3	5	8.5	5	8.6
NaOH + NaCl	0.6	7.0	2	8.0	2	8.3	2	8.4
NaOH + Na_2SO_4	0.3	5.7	2	6.8	2	7.8	3	8.1

and all the significant points in table 9, there are 28 comparisons of this kind. In 22 cases the mixture is intermediate in effect. Of the six exceptions, two occur in the case of the 25 per cent stimulation point with Na_2CO_3 and Na_2SO_4 where the figures are very small and the differences probably not significant. The other four exceptions all occur in NaH_2PO_4 and here the explanation is very clear. The solutions of NaH_2PO_4 alone were highly acid

(pH 4.5 to 5.3) and the mixture with NaOH neutralized this high acidity (see table 9, columns 3b, 4b, 5b, 6b).

In general, then, and with the obvious and entirely reasonable exception of NaH_2PO_4, the effect of mixing NaOH with a neutral salt in equal molal proportions is a simple weakening of the effect of the NaOH by a less active diluent.

In order to make the relative effects of the various anions a little clearer, table 10 has been prepared from the data in table 9, using the effective concentration of NaOH as unity and computing the effective concentration of each salt mixture in terms of the corresponding effective concentration of NaOH.

Column 2a of table 10 again makes it clear that the stimulating effect of the various salt mixtures varies very little with the anion involved. All the concentrations producing a 25 per cent increase are of about the same order of magnitude with the exception of NaH_2PO_4 which is less potent than the rest. On the whole, however, it would seem that the Na ion is the chief factor in this primary stimulation.

The inhibiting effects, as indicated in columns 3a, 4a and 5a are, on the other hand, highly variable. The compounds studied divide themselves into two major groups. NaOH, Na_2CO_3 and Na_3PO_4, with their mixtures, are all highly potent and of approximately the same order of magnitude. The other five salts (NaH_2PO_4, $NaHCO_3$, Na_2HPO_4, NaCl and Na_2SO_4) with the mixture of NaOH and NaH_2PO_4 are much less potent, requiring a concentration usually twenty to one hundred times that of sodium hydroxid to produce an equivalent effect.

VI. EFFECT OF HYDROGEN ION CONCENTRATION UPON THE POTENCY OF THE SALTS AND SALT MIXTURES

In order to explain the difference between the effect of the various sodium compounds studied, we must obviously turn to the effects of anions or undissociated salts and the first factor to be considered is naturally that of hydrogen ion concentration. The figures in table 11, rearranged from those in table 10 clearly indicate that this factor will materially help to explain the results obtained.

Inspection of table 11 shows that the salt mixtures above the heavy line which are all of about the same order of potency as NaOH are generally highly alkalin solutions. There are only 6 out of 27 pH figures in this part of the table under pH 8.0 and only one figure under pH 7.7 (mixture of NaOH + Na_2SO_4). On the other hand, in the lower part of the table, out of 18 pH figures only three exceed pH 8.0 (all of them in the case of Na_2HPO_4).

TABLE 11

Relative potency of various salts and salt mixtures in relation to hydrogen ion concentration; relative concentration producing equivalent effects

SALT	MAXIMUM		CROSS-OVER		50 PER CENT DECREASE	
	Concentration	pH	Concentration	pH	Concentration	pH
NaOH	1	8.0	1	8.4	1	8.5
NaOH + Na_2PO_4	3	8.4	2	8.7	2	9.0
NaOH + Na_2CO_3	1	7.8	2	8.3	2	8.4
NaOH + $NaHCO_3$	2	7.7	2	7.8	2	7.8
NaOH + NaCl	2	8.0	2	8.3	2	8.4
Na_2CO_3	4	8.2	2	8.6	3	8.8
NaOH + Na_2SO_4	2	6.8	2	7.8	3	8.1
Na_3PO_4	6	8.4	4	8.5	5	8.8
NaOH + Na_2HPO_4	4	8.3	5	8.5	5	8.6
NaH_2PO_4	10	5.2	14	4.8	20	4.5
$NaHCO_3$	15	7.9	17	8.0	22	8.0
Na_2HPO_4.....	20	8.1	54	8.4	55	8.4
NaCl	25	5.7	57	5.7	61	5.7
NaOH + NaH_2PO_4	20	7.5	97	7.7	89	7.7
Na_2SO_4.......	25	5.5	57	5.5	111	5.5

It would seem that the potency of the various compounds in their effect upon bacterial viability can, in general, be explained as due to the combined effect of their sodium content and their hydrogen ion concentration. Na_2HPO_4 seems, however, less potent than would be expected on the basis of its considerable alkalinity while mixtures of NaOH with $NaHCO_3$ and Na_2SO_4 respectively are more potent than would be predicted.

In order to test the adequacy of the assumption that via-

bility in our experiments was determined primarily by these two factors,—sodium concentration and pH,—figure 2 was prepared. In this graph, ordinates represent logs of molal concentration of sodium and abscissae represent pH values. The average results for all of the 158 salt mixtures studied in Dolloff medium with unwashed cells have been plotted on this graph, without reference to the particular salts concerned. At

TABLE 12

Distribution of viability results in different areas of figure 2

VIABILITY	AREA I	AREA II	AREA III	AREA IV	AREA V
Reduction			3	5	42
Less than 50 per cent decrease . .		2	13	42	5
50 to 100 per cent increase		3	24	1	
100 to 500 per cent increase		12	3		
Over 500 per cent increase	3				
Total..	3	17	43	48	47

TABLE 13

Analysis of deviation from primary relationship

	AREA II	AREA III	AREA IV	AREA V
Deviations in excess of value characteristic of area		$Na_2HPO_4 + NaOH$ $Na_2HPO_4 + NaOH$ $NaH_2PO_4 + NaOH$	NaCl	NaOH $Na_2HPO_4 + NaOH$ $Na_3PO_4 + NaOH$ $Na_3PO_4 + NaOH$ Na_3PO_4
Deviation below value characteristic of area	$NaHCO_3$ $NaHCO_3$ $NaHCO_3$ $NaHCO_3$ Na_2HPO_4	$Na_2SO_4 + NaOH$ Na_2CO_3 $NaHCO_3 + NaOH$	NaH_2PO_4 Na_2CO_3 $NaHCO_3 + NaOH$ Na_3PO_4 $Na_3PO_4 + NaOH$	

the point corresponding to the Na concentration and pH for each salt or mixture a figure representing the number of bacteria present has been written down, expressed as usual in per cent of the number present in the control Dolloff medium without salt. Thus, in table 1, 0.001 M NaCl had a pH of 5.7 and gave 22 per cent more bacteria than did the Dolloff control. Therefore opposite the ordinate 0.001 and above the abscissa 5.7 the figure +22 is written down in figure 2.

The results of this method of graphic treatment are striking, a concentration of high counts in the upper right hand section of the chart, with counts decreasing in all directions from this center, being clearly obvious. The closeness of this relation was tested by drawing lines round those areas in the graph which include similar bacterial counts. These areas are indicated in figure 2 by the Roman numbers I to V and the remarkable evenness of distribution which results is indicated in table 12.

This type of diagram is somewhat unusual in biological analysis, though familiar in principle to engineers. It is of very great value since it makes it possible to observe the simultaneous influence of two more or less independent variables. Lawrence Henderson has applied a somewhat similar principle in his studies of blood chemistry and has shown how much more significant such an analysis may be than the ordinary approach which considers but one variable at a time. Figure 2 may be interpreted in terms of a topographical map. If a layer of cardboard half an inch thick were over the whole chart, a second layer of cardboard cut out to correspond to area IV, and placed on the first, a third layer cut out to correspond to area III and placed on top of the second, and so on,—we should get a relief map of a mountain with its peak in area I, a very sharp declivity toward high pH values, a more moderate but still marked declivity toward high Na values and a very gentle slope toward low Na and pH values. The fact that the figures on the chart group themselves so smoothly on these slopes and that so few irrelevant peaks and valleys appear makes it certain that the major influences at work are two only, Na concentration and pH.

It seems evident that with a Na concentration of about 0.10 M and a pH of 7.5 conditions are most favorable for viability. All of the three observations in area I gave counts 500 per cent in excess of the control.

Area II surrounds area I, extending from 0.04 M to 0.60 M, Na at optimum pH and extending at optimum Na concentration over a wide pH range, from 5.5 to 8.3. The areas are in general somewhat spindle-shaped, as would be expected, the zone of pH tolerance being widened at optimum Na concentration and the zone of Na tolerance widened at optimum pH.

Area III adjoins area II, extending to lower Na and pH values. It does not extend to the upper right zone of the chart because at high Na and pH values there is a sharp critical point, a very slight further increase in either factor changing a strongly stimulating to an inhibiting effect. Area III extends down to a Na concentration of 0.005 M and at optimum Na concentration extends down to a pH of 5.1. In this area, 24 observations show an increase of 50 to 100 per cent, 13 an increase of less than 50 per cent, 3 an increase of 100 to 500 per cent and 3 a decrease (see table 12).

Area IV again surrounds area III, except in the upper right hand zone. It includes the lower Na concentrations in the favorable pH zone and the more acid solutions in the favorable Na zone. Forty-two out of 48 observations in this area show increases of under 50 per cent in bacterial counts, 1 a greater increase and 5 a reduction.

Finally, area V includes (a) the very high Na concentrations (over 0.6 M); (b) the solutions of moderately high Na (0.3 to 0.5 M) plus moderately high pH (8.0 to 8.3); (c) the highly alkalin solutions (over pH 8.3); and (d) solutions of moderately high Na concentration (0.2 to 0.5) combined with acid reaction (pH under 5.0). Of 47 observations in this area, 42 show reductions in bacterial count, obviously due either to a highly unfavorable Na or pH effect or to a combination of a moderately toxic pH with a moderately toxic Na concentration. Only 5 show a slight increase.

It would appear therefore that the two factors of Na-concentration and pH will serve to account for most of the viability phenomena observed.

Such a chart as figure 2 makes it possible not only to establish general relationships but also to study exceptions, which may reveal factors of secondary but real importance. Reference to table 12 shows that in area II there are 5 observations which deviate from the general values, characteristic of the area, 3 observations showing a 50–100 per cent increase and 2 an increase of under 50 per cent. In area III there are 6 observations deviating markedly from the characteristic figure, 3 showing a reduction and 3 an increase of over 100 per cent. In area IV there are 6

marked deviations, 1 above and 5 below the characteristic figure, while in area V there are 5 marked deviations above the characteristic figure. These 22 deviations are analyzed in table 13. It will be noted that of 9 deviations above the characteristic range of any area, 7 occur in phosphate solutions. Of the 13 deviations downward, 8 occur in carbonate solutions and 4 in phosphate solutions. It would seem probable then, that in addition to the primary effects of Na and pH, phosphate solutions tend to be stimulating in their effect and carbonate solutions inhibitory. The assumption of some specific favorable influence of phosphates is borne out by an analysis of the very high points in figure 2. All of the nine points in areas I and II showing increases of over 200 per cent were observed in the mixture of Na_2HPO_4 and NaOH. Furthermore, of the six other observations in area II showing increases of 100 to 200 per cent, 4 were in Na_2HPO_4. Finally, of the only 3 observations lying outside of areas I and II but showing an increase of over 100 per cent, 1 was in NaH_2PO_4 + NaOH and 2 in Na_2HPO + NaOH. It would appear, therefore, that while the mixture of NaH_2PO with NaOH does give a combination of Na concentration and pH which lies just in the area which our graph shows to be most favorable, there must also be some specific favorable influence of the phosphate radicle itself. This effect is not generally manifest with Na_3PO_4 on account of the unfavorably high concentration of sodium present.

<div align="center">VII. CONCLUSION</div>

The data presented would seem to warrant the following conclusions:

1. Reasonably uniform and significant results may be obtained by the study of the viability of *Esch. coli* in a simple synthetic medium (Dolloff's) in the presence of various electrolytes. Previous washing of the cells used for inoculation increases their sensitiveness to the electrolytes.

2. All electrolytes studied (NaOH, NaCl, $NaHCO_3$, Na_2CO_3, Na_2SO_4, Na_2HPO_4, NaH_2PO_4, Na_3PO_4 and mixtures of all of these salts with NaOH) exhibit the same general qualitative effect. All stimulate the growth of bacteria in low concentration and inhibit it in higher concentration.

3. The stimulating effect of these electrolytes in low concentration appears to be a direct function of their Na content,—a concentration of Na of between 0.001 and 0.020 molal strength being most favorable, unless other factors supervene.

4. The inhibiting effect of higher concentration of electrolytes is due to two factors,— sodium concentration and hydrogen-ion concentration. These two factors appear to explain most of the observed phenomena.

5. Maximum stimulation occurs with a sodium concentration of 0.10 M and a pH of about 7.5.

6. Decrease of Na concentration and decrease of pH value below this optimum results in a gradual decrease of the stimulating effect. An increase in Na or pH causes a much more rapid change from stimulation to inhibition.

7. Na concentrations above 0.60 M and pH values above 8.3 are inhibitive and cause a decrease instead of an increase in bacterial numbers, as compared with the salt-free control.

8. If Na concentration and pH value be within a favorable range, the presence of the phosphate radicle exerts a distinctly stimulating influence.

9. It is suggested in view of these facts that two major factors govern bacterial viability under the conditions of these experiments. The first is the cation concentration and according to the theory of Winslow and Dolloff (1928) the effect of all cations is qualitatively alike and differs only in degree. The second factor is the pH which, with a similar cation, is determined by the anion. The phosphate radicle has a specific stimulating effect of its own if cation concentration and pH be optimum.

10. We suggest that in studies of salt action upon bacteria (and perhaps other organisms) the attempt should be made to explain observed results on such simple bases as those outlined above. Assumptions of "specific salt action" and of "antagonism" and of the influence of one salt or alkali or acid upon the toxicity of another need not be made except where this simpler explanation fails.

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STUDIES ON OXIDATION-REDUCTION IN MILK

I. OXIDATION-REDUCTION POTENTIALS AND THE MECHANISM OF REDUCTION[1]

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The recent work of Clark and his co-workers (1928, I–X) makes desirable a reinterpretation of the meaning of dye reduction in milk, and provides a new and excellent avenue of approach to the study of oxidation-reduction phenomena in this biological fluid.

Oxidation is defined as the process in which a substance takes up positive, or parts with negative, charges, while reduction is the process in which a substance takes up negative or parts with positive charges. These electronic changes may be followed in milk potentiometrically. Clark (1925), Thornton and Hastings (1927, 1928), and Thornton (1927, 1929) have reported such experiments. The present paper is a fuller presentation of the work reported earlier by the latter two authors.

OXIDATION-REDUCTION POTENTIALS

If an electrode of one of the regal metals is immersed in milk and connection is made to a potassium chloride calomel half-cell through a potentiometer and the circuit completed through a potassium chloride agar bridge, the potential difference between the milk and the calomel half-cell is easily measured. By a simple computation this potential may be referred to a standard zero, the normal hydrogen electrode. The difference in potential between the normal hydrogen electrode, the potential of which is

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arbitrarily assumed to be zero, and the milk is termed E_h.   A schematic representation of these relationships is given below. In the illustration chosen the observed E.M.F. was 0.4864 volt, the unknown being negative to the calomel cell.   The E_h is, therefore, -0.15 volt.

E_h scale in volts
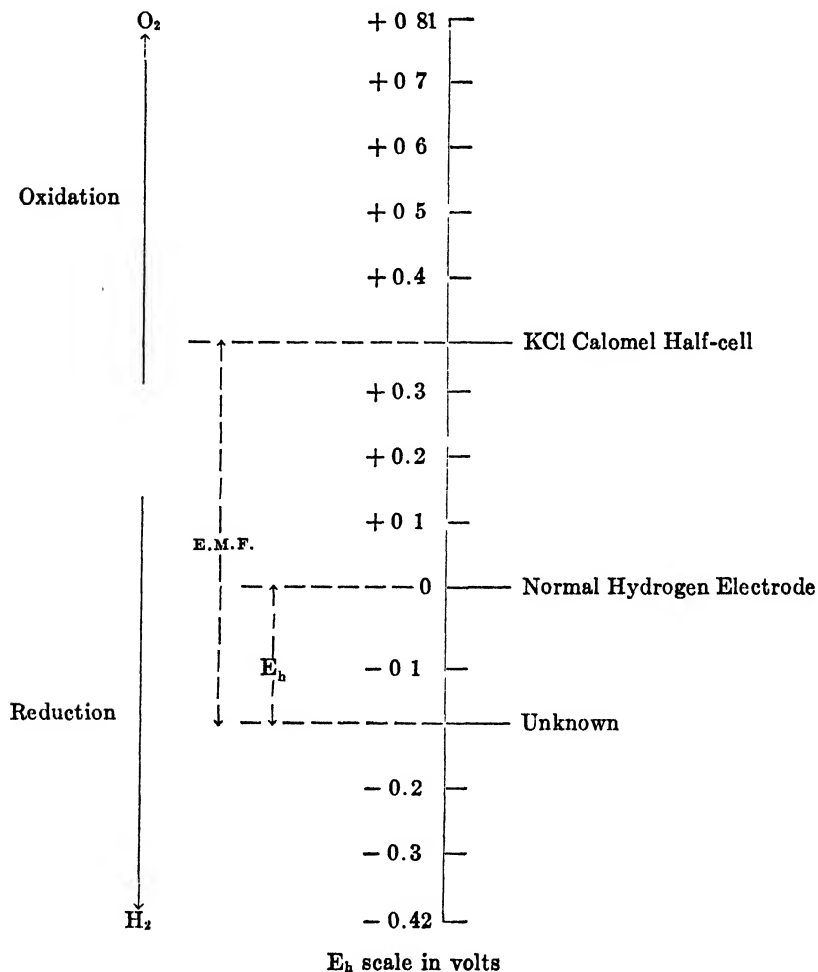
For the work reported in this paper a constant temperature bath operated at 37.5°C. $\pm 1°$ was fitted with hydrogen leads to accommodate 6 samples.   Therefore the pH values of all of our

samples were determined at frequent intervals. Wires from 6 electrodes led through a 6-way switch to a Leeds and Northrup Type K potentiometer. Platinized-platinum electrodes were used in hydrion determinations and gold-plated platinum electrodes for oxidation-reduction potential determinations. A 0.1 N potassium chloride calomel half-cell was used. Connection was made through a saturated potassium chloride liquid junction
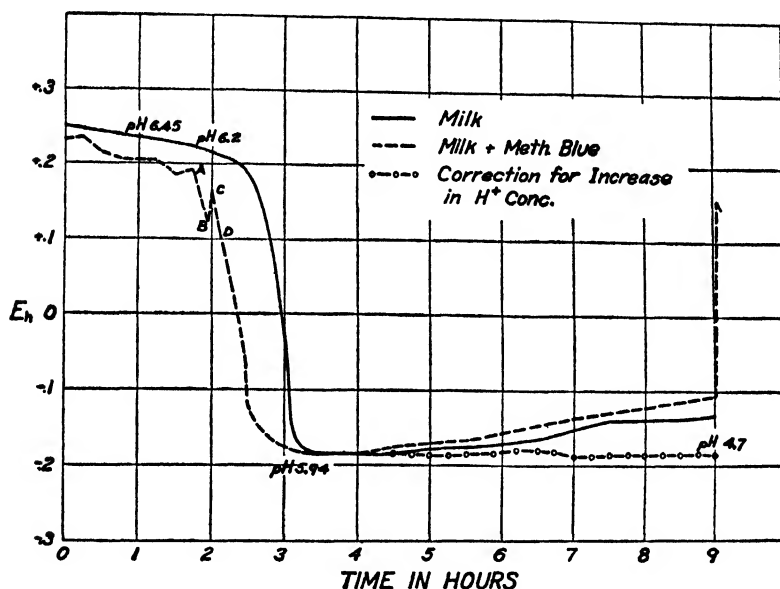
FIG. 1. POTENTIAL-TIME CURVES OF A SAMPLE OF MARKET MILK WITH AND WITHOUT THE ADDITION OF METHYLENE BLUE

by means of saturated potassium chloride agar bridges. All readings of E.M.F. are reported in volts in terms of E_h.

The oxidation-reduction potentials of a number of milk samples were followed at few minute intervals. The results of one such experiment are given graphically in figure 1. The solid line represents the potential changes in the milk alone. In the case of the broken line the standard amount of methylene blue (1 part of dye to 200,000 parts of milk) had been added. This latter milk was shaken at half-hour intervals as gently as possible to

incorporate only the minimum amount of oxygen and at the same time prevent the rising of the butter-fat. These curves show distinctly the effect of shaking upon the reduction potential and time. This effect will be given full consideration in a later paper. At point A no reduction of the methylene blue could be observed, while at point B the milk had turned white. The vessel was then shaken and the blue color reappeared. The potential was then read as at C. Complete visual reduction had again taken place at D. Shaking was then discontinued till the ninth hour. This last shaking caused the potential immediately to swing almost to the positive extreme, and a faint blue color reappeared in the milk. A further reading within five minutes showed the potential to be changing rapidly toward the negative side. When the changes in the potentials as read with the hydrogen electrode were subtracted from the potential values as read with the gold-plated electrode and the values plotted, the circle-line curve was obtained. This suggests that the upward trend of the curve in the last five hours of the experiment was due to the increase in the hydrion concentration.

Examination of this figure shows that the dye decolorized in a zone about 0.1 volt more positive than the theoretical for methylene blue at this pH (see Clark, 1925). This seems to indicate salt effect or the influence of another oxidation-reduction system or systems. This effect is not constant but varies in different milks, and we wish to point out that, at present, caution should be used in interpreting reduction intensities in organic complexes in terms of potential on the basis of dye reduction. We have observed complete visual reduction of methylene blue in different milks at E_h values as low as +0.075 volt (rH 14.5) and as high as +0.225 volt (rH 19). rH is defined as the logarithm of the reciprocal of the hydrogen pressure.

Figure 2 represents a similar experiment except that the milk containing the dye was left undisturbed until after decolorization had taken place. The sharp inflection in the solid line was caused by the changing of electrodes at this point. Reduction of the dye had taken place at A. The potential change was so rapid and the end-point of reduction so indefinite that the

points of reduction are, perhaps, only approximate. At *B* the milk containing the methylene blue was shaken with the result seen in the graph. The blue color returned to the milk and this had again disappeared when *C* was reached.

The positive E_h limits of all the milks examined lay between +0.2 and +0.3 volt. This corresponds closely to the limits reported by Clark and others for milk and some other biological fluids. The negative limits reached by all our milks were re-
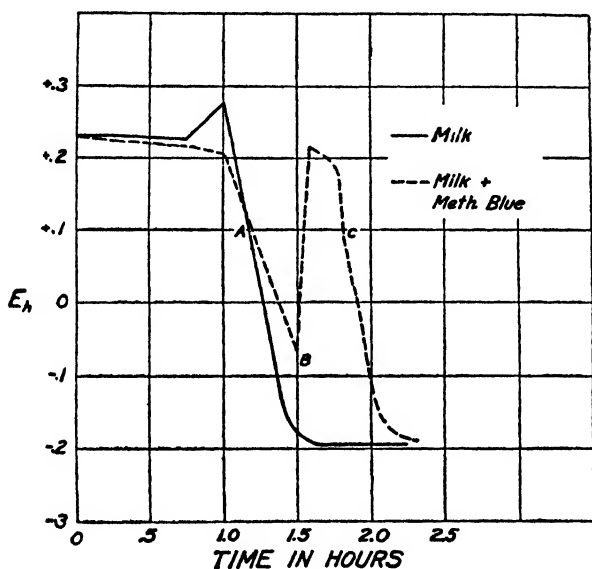
FIG. 2. POTENTIAL-TIME CURVES OF A SAMPLE OF MARKET MILK WITH AND WITHOUT THE ADDITION OF METHYLENE BLUE

markably uniform, approximating E_h −0.2 volt. This is the limit of reduction potential reached by a pure culture of *Streptococcus lactis* growing in milk as reported by Clark (1926), and suggests that the predominating influence in our milks was that of the lactic bacteria. Very little work, however, has been done on the oxidation-reduction potentials of pure cultures of bacteria, so that a definite conclusion cannot be drawn.

"A solution may be said to be *poised* when it tends to resist change in E_h on addition of an oxidizing or reducing agent."

These curves reveal a slight *poising* effect of the methylene blue in milk. This effect is so small as to be almost negligible when the standard dye concentration is used.

<div align="center">

THE MECHANISM OF REDUCTION

The rôle of hydrogen

</div>

The accepted equation for the reduction of methylene blue to methylene white is given below. It will be seen from this equation that the reduction of this dye involves no change of oxygen, since there is no oxygen in the methylene blue molecule. Two atoms of hydrogen are concerned, however. One atom withdraws the chlorine from the basic terminal nitrogen. A shifting of the double bonds takes place leaving the bridging nitrogen unsatisfied. The other atom of hydrogen links up with this nitrogen forming methylene white.
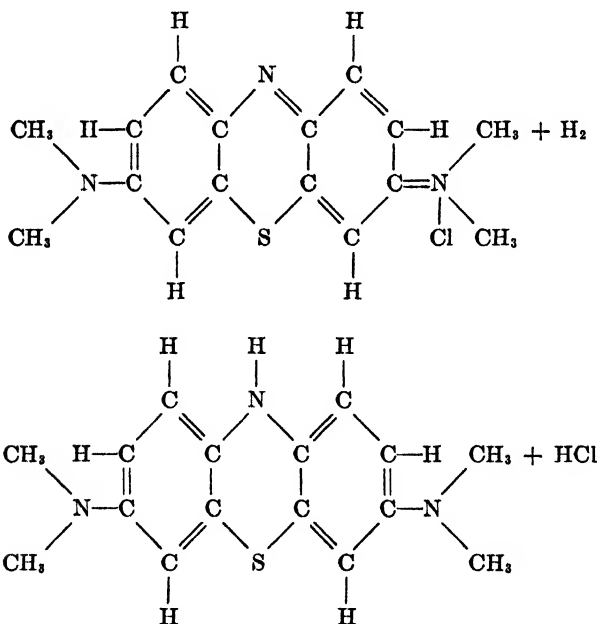
This immediately suggests the interesting problem of the source of the hydrogen involved which has been a matter of

controversy for years.   Lactose, which is present in milk to the extent of about 5 per cent, is a reducing sugar and must receive consideration as a contributing agent in the reduction of dyes in milk.   Of recent years attention has been focused upon a group of compounds which have been called "metabolites."   Hopkins (1921) believes glutathione to be the hydrogen donator and acceptor in oxidation-reduction processes in animal tissues. He states that glutathione is a compound of cysteine and glutamic acid with a free S—H group.   Harding and Cary (1926) have demonstrated this compound in the blood of the cow in larger quantities on entering than on leaving the udder.   Viale (1925) and others have shown that other such compounds having the sulfhydryl group enhance the reducing powers of milk.   Thunberg (1925) found that succinates effect reduction potentials. The power of succinates and citrates to aid in reduction in milk has been demonstrated by Barthel (1925).   He believes the citrate in the milk to be the hydrogen donator according to the following equation:

$$
\begin{array}{c}
\text{COOH} \\
| \\
\text{CH}_2 \quad \text{OH} \\
| \quad / \\
\text{C} \\
| \quad \backslash \\
\text{CH}_2 \\
| \quad \text{COOH} \\
\text{COOH}
\end{array}
\quad - 2H + H_2O =
\begin{array}{c}
\text{COOH} \\
| \qquad \text{OH} \\
\text{CHOH} / \\
| \quad / \\
\text{C} \\
| \quad \backslash \\
\text{CH}_2 \\
| \quad \text{COOH} \\
\text{COOH}
\end{array}
$$
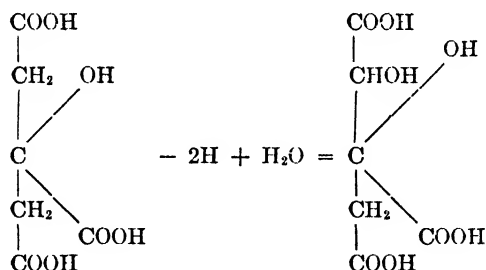
This reaction, he thinks, is catalyzed by the milk salts.   His experiments showed increasing acceleration of the reduction of methylene blue in milk on the addition of increasing amounts of sodium citrate or succinate.   Quastel (1926)

examined 103 substances as possible donators or acceptors of hydrogen (using the methylene blue technique) in the presence of bacteria (B. coli) and of these 56 are activated.   That is, of the 56 substances some reduce methylene blue, while others oxidize leucomethylene blue under conditions (pH 7.4 and 45°) when they are apparently quite inactive in the absence of the organism.

Clark (1926) published potential-time curves showing the effect of the addition of such metabolites as sodium succinate and glutathione upon the potentials of suspensions of washed yeast cells.

To study the effect of the addition of cysteine upon the reduction of methylene blue in milk two tubes of the same milk containing methylene blue were incubated at 37.5°C. for thirty minutes at which time cysteine was added to one tube. The
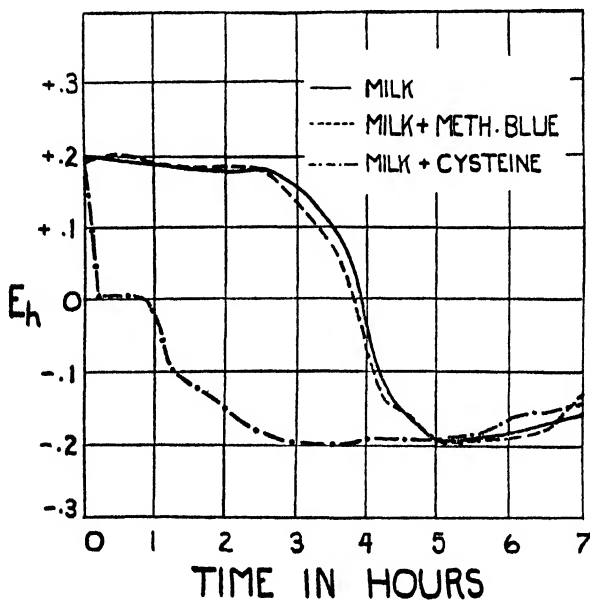
FIG. 3. POTENTIAL-TIME CURVES OF A SAMPLE OF MARKET MILK WITH AND WITHOUT THE ADDITION OF METHYLENE BLUE AND OF CYSTEINE

methylene blue in this tube decolorized in another hour, or in one and one-half hours from the start of incubation. The methylene blue in the tube containing no cysteine reduced in nine hours from the start of incubation. Figure 3 shows the effect of the addition of cysteine in a concentration of 1 part cysteine to 1000 parts milk upon the potentials in a sample of milk. Attention is called to the uniformity of the negative limits of potential. If the limit is due to the influence of *Streptococcus lactis,* then

this influence is greater than that of cysteine in this concentration. Table 1 shows the results of another experiment in which a number of substances were added to duplicate tubes of milk and methylene blue reduction times noted.

The question, therefore, of the hydrogen source in the reducing processes in milk is not settled as yet. It seems probable that a number of the constituents of the milk are concerned. For the practical application of the methylene blue reduction test for quality in milk this is of no great importance for, as will be shown later, the inherent reducing "capacity" of milk is not over-taxed by the concentration of dye used in this test.

TABLE 1

The effect of the addition of a number of substances upon the reduction time of methylene blue in milk

SUBSTANCE	CONCENTRATION	REDUCTION TIME
Sodium citrate	1:1000	7:30
Sodium citrate	1:500	7:30
Sodium nitrite	1:1000	9:30
Sodium nitrate	1:1000	7:30
Cysteine	1:1000	2:00
Control		7:45

Note: Reduction times are reported in hours and minutes. Thus 7:30 means 7 hours and 30 minutes.

The rôle of oxygen

It is a common observation that the blue color returns to reduced methylene blue milk mixtures on shaking with air. That this is due to the oxygen in the air is proved by the fact that neither hydrogen, nitrogen, nor carbon dioxide will cause the oxidation of methylene white to methylene blue in milk, while oxygen will do so.

This oxygen relationship may be demonstrated in a number of ways. On heating an alkaline solution of a reducing sugar, to which methylene blue has been added, reduction takes place. On cooling and shaking with air, the blue color returns. This may be repeated many times. If the unheated solution is left exposed

to the air in a thin layer, reduction does not take place.   If, however, diffusion of atmospheric oxygen into the liquid is prevented by a paraffin or vaseline seal, reduction will take place at room temperature.   The dye is also reduced in such a solution in an uncovered test-tube where the surface exposed to the air is small in proportion to the volume of the liquid.   Any of these reduced solutions of sugar will regain their blue color on shaking with air.   Thus the sugar solution consumes oxygen and that oxygen bears an important relationship to reduction.

If a sterile methylene blue milk mixture is allowed to stand in a thin layer with the surface exposed to the air, reduction does not take place.   If the same mixture is placed in test-tubes and sealed with vaseline, or even with the normal cream layer of

TABLE 2

The effect of exhaustion upon the reduction time of methylene blue in milk

EXHAUSTION TIME	REDUCTION TIME
minutes	
0	4:05
2	2:50
15	1:15
30	0:45

whole milk, and incubated at room temperature, reduction ultimately takes place.   If a sterile mixture of methylene blue and skim milk is placed in test-tubes and the test-tubes sealed off in a flame, reduction of the dye will take place.   The reduction period may be three or even six months depending upon the ratio of the volume of milk and of air sealed above the milk.   That the milk has consumed the oxygen, thus allowing reduction, is shown by the quick return of the blue color if the tubes are opened and the contents shaken with air.

In one experiment, the reduction time of methylene blue in a sample of milk was found to be forty-five minutes.   The reduction time in a duplicate sample through which oxygen had been bubbled for a few moments was one hour and forty-five minutes.   Harvey (1919) has shown that the reduction times

in the Schardinger reaction vary with the oxygen content. We have found the same to be true in the reduction test as is shown in table 2. For this experiment quantities of the same milk were exhausted by means of a waterpump and the reduction times noted.

Barthel's experiments with deaerated raw and heated milks and the work reported later in this paper with similar milks prove without question that oxygen is an important factor in dye reduction by milk. Scrutiny of figure 1 of this paper will show the effect of oxygen upon the reduction potentials in milk and that these potentials depend, in the main at least, upon the oxygen content of the milk.

In his series of papers on oxidation-reduction Clark reports that certain of the indolphenols will reduce immediately in milk. Drs. Clark and Cohen have very kindly supplied us with a number of their dyes and we have confirmed these observations with two of the indophenol indicators. These dyes were reduced almost immediately, even in fresh milk of low bacterial content. The ranges of reduction intensity within which these indicators pass from the oxidized to the reduced state lie outside the range of reduction intensity in milk toward the positive end of the E_h scale. Clark reports reduction of 2-6 dichlor-indophenol by a suspension of washed yeast cells "while a vigorous stream of air was being passed through the suspension." This suspension was unable to reduce methylene blue appreciably under anaerobic conditions.

Other dyes, reducing over a potential range more negative than that of methylene blue, will be reduced in milk in greater time than that necessary for the reduction of methylene blue and reduction will usually be in the order in which these dyes fall upon the E_h scale.

We have found that when the potential has reached the negative limit minute amounts of oxygen will cause the potential to swing rapidly and markedly toward the positive side (fig. 1). This sensitivity toward oxygen appears to increase with the fall in potential. If, now, we assume that the bacteria are reproducing every half hour, then the growth of the bacteria within

any given half hour represents one-half of the total oxygen-consuming power of the bacteria up to the end of that time. It is improbable that this assumption is ever correct.   In one instance, however, plate counts made every half hour roughly doubled in that period of time.   The main drop in potential from the positive to the negative side usually takes place in about one half hour.   Therefore, the oxygen-consuming power of the bacteria during this period must be relatively great.   This has led one of us (Thornton, 1927) to conclude that, while small amounts of oxygen are probably present at the time of decolorization of methylene blue, anaerobic conditions obtain when the lower limit of potential is reached.   This opinion we now wish to revise.   It seems more satisfactory to suppose that anaerobic conditions are not reached at the negative potential limit as shown in figures 1, 2 and 3, but that an equilibrium is established at this point and the oxygen content of the milk remains constant. We are not in a position to give an opinion as to the influence of oxygen upon the potential of aqueous solutions which have reached a reduction intensity more negative than those reported in this paper.   Clark (1924) states

In a case where we allowed bacterial reduction of indigo carmine to proceed to 80 per cent reduction we measured the potential electrometrically and calculated therefrom the oxygen pressure.   It came out 10^{-36} atmospheric.   Using the data of Millikan on the number of molecules per gram mole of gas we calculate that less than one discrete individual molecule of oxygen was present at equilibrium in 10^{13} liters of the culture.

It seems apparent, therefore, that the reduction of methylene blue by a culture is not necessarily an indication of anaerobic conditions, but merely that a certain partial pressure of oxygen has been reached.   This possibly explains some of the unsatisfactory results bacteriologists have experienced when using this dye as an indicator for oxygen in anaerobic culture work.   It is probable that the effect of oxygen upon the reduction intensities of different milks and different media is not quantitatively identical.

The theory of Wieland (1922) that the dissolved oxygen be-

comes the acceptor for the hydrogen is the popular explanation today for the disappearance of oxygen in biological reductions. According to this theory hydrogen combines with the oxygen to form first H_2O_2 and then H_2O as represented by the following equations:

$$O{=}O + H_2 = HO{-}OH$$

$$\begin{array}{ccc} OH & H & OH_2 \\ | & + & = \\ OH & H & OH_2 \end{array}$$
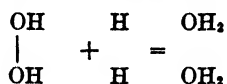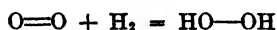
The rôle of enzymes

The early workers believed the phenomenon of methylene blue reduction in milk to be due to enzyme action, hence the name "Reductase Test." The reductase was supposed to be elaborated by the growing bacteria. This is well illustrated by the following conclusion of Fred (1912):

Reductases are formed by the growth of microörganisms and do not occur in milk when first drawn. The reduction of methylene blue, free of formalin, is very complex and is no doubt aided by the changes of matter during assimilation. Very probably both intracellular and extracellular products take part in the reduction.

Most workers have failed to recognize the relationship of oxygen to the reduction of dyes in milk and in their experiments provided no substitute for the oxygen-consumption of the bacteria when these were not present or were present only in small numbers. The enzyme theory of reduction has not been a satisfactory one and leaves unexplained such phenomena as the reduction of dyes in sterile milk and other bacteriological media.

Burri and Kürsteiner (1912) reported reduction in fresh milk of low bacterial content in which bacterial growth was inhibited by antiseptics. They concluded that this reducing power is inherent in the milk and is of non-bacterial origin. Barthel (1917) observed reduction of methylene blue in freshly autoclaved milk within 1 hour and 50 minutes. This precludes entirely a bacterial enzyme theory. There remains, however, the possibility that the reducing power of the milk is created or increased be-

cause of chemical changes of the milk constituents during the heating process. The creation of a reducing property in milk by heating seems out of the question in the face of further experiments by Barthel in which he removed the oxygen from fresh raw milk of low bacterial content with hydrogen, carbon dioxide, and nitrogen and obtained reduction, in one case in thirty minutes.

With a similar use of molecular hydrogen or carbon dioxide we have observed reduction in thirty minutes at 37.5°C. in fresh raw milk of low bacterial content. Using the same technique on a freshly autoclaved nonaerated milk (except for the aeration consequent upon the careful addition of sterile but aerated methylene blue solution) reduction was complete in ten minutes. In our experiments this reduction time includes that time necessary to wash out the residual oxygen as well as the time taken for the actual reduction of the dye. We used 10 cc. of milk while Barthel used 40 cc. Apparently an inherent reducing power is present in milk at the time it is drawn from the udder. The heat of autoclaving removes sufficient oxygen to allow the potential to pass through the range of methylene blue. Diffusion of atmospheric oxygen into the milk on cooling causes this potential to swing to the positive side again and the leucobase undergoes oxidation. The assumption that heating increases the reducing power of milk is, therefore, not necessary in order to explain the reduction of dye in these experiments.

It is our opinion that failure to recognize the reducing properties of various media in which bacteria or other cells are suspended and insistence on attributing reduction solely to the suspended cells are instrumental in masking much that would aid in explaining biological reductions. Clark (1926) in reporting on reduction potentials in cell suspensions says

It is well known from the work of Battelli and Stern, Thunberg, Harden and Morris, and others that the ability of a suspension of cells to reduce methylene blue is almost completely removed by exhaustive washing with water. We have followed the potentials developed when washed cells (yeasts, muscle and liver) are suspended in a deaerated buffer in the electrode vessel already referred to. The more exhaustive the

washing, the less definite become the potentials, until, for example, suspensions of six times washed yeast or washed muscle hold the electrode only most erratically.

It is likely that all organic complexes consume oxygen. It seems highly probable that any medium, in, or on, which bacteria or yeast can grow, will also possess reducing powers. Small amounts of this medium will be transferred to the buffer solution in which the cells are suspended and the quantity will diminish with repeated washings. Among the cell constituents there are, without doubt, compounds not entirely dissimilar to those making up the common laboratory media. There is a possibility that some of these diffuse into the surrounding buffer solution increasing the reducing properties of that solution. It is probable that repeated washing would lessen these water-soluble and diffusable constituents. If this is so, then the problem of biological oxidation is, to some extent at least, simplified.

Every bacteriologist has noticed that litmus milk is reduced as it comes out of the autoclave and quickly becomes colored again as it cools. If oxygen diffusion is prevented by a seal of vaseline or butterfat, a slight purpling will take place coincident with cooling. This color will disappear in a few hours or a few days when the milk has used up the oxygen which entered while the seal was hot and in a liquid state. Litmus reduces over a potential range more negative than that of methylene blue. Milk so treated will also reduce potassium indigo tetrasulphonate and will cause the first irreversible reduction of janus green but will not reduce the pink safranin compound which results, nor will it reduce safranin. The potential ranges over which these dyes reduce are more negative than that of the methylene blue-methylene white reaction.

Other bacteriological media will reduce certain of these indicators if the dissolved oxygen is driven off by such means as heating. Table 3 shows the reducing power of plain and lactose nutrient broth and plain and lactose nutrient agar. Tubes of the different media containing the various dyes were autoclaved at 15 pounds pressure and a reading made immediately. In two

hours another reading disclosed the fact that reoxidation of all the dyes except janus green had taken place during cooling. Doubtless oxygen had entered the media while the vaseline seal was liquid and convection currents possible. This oxygen was consumed by the media after incubation at room temperature for seventy-two hours. The janus green was considered reduced on the appearance of the red safranin compound. This reaction is thought to be irreversible and will be considered in detail in a later paper. The red compound was in no case reduced. Unless protected by a seal, reduced conditions are more easily

TABLE 3

The reduction of various dyes in plain and 1 per cent lactose nutrient broth and plain and 1 per cent lactose nutrient 1.2 per cent agar at pH 6.8 after heating at 15 pounds pressure

| | BROTH | | | | AGAR | | | |
| | Plain | | Lactose | | Plain | | Lactose | |
	Immediately	After 72 hours	Immediately	After 72 hours	Immediately	After 72 hours	Immediately	After 72 hours
2.6 dichloro phenol indo phenol............	+	+	+	+	+	+	+	+
Ortho cresol, 2.6 dichloro indo phenol.....	+	+	+	+	+	+	+	+
Methylene blue...........................	±	±	+	+	+	+	+	+
Janus green..............................	−	±	+	+	+	+	+	+
Litmus...................................	−	−	±	±	±	±	±	+

Note: + means reduced; − means not reduced; ± means partially reduced.

maintained in agar media than in liquid media. Fred reports reduction of methylene blue in all parts of a tube of agar medium upon which a culture was growing, and concludes that the bacteria produced an exoreductase. In the light of present knowledge it seems more probable that the bacteria and the medium consumed the oxygen and that the dye was reduced by constituents of the medium.

The reduction potentials of plain nutrient broth have been studied by Coulter (1928) and Dubos (1929). The latter used the colorimetric method. The former, who measured the po-

tentials electrometrically, found that the initial E_h values of aerated broth lay between $+0.15$ volt and $+0.25$ volt, which is remarkably close to the positive E_h limits of fresh milk. The negative potential limits reached were between E_h -0.05 volt and -0.06 volt. The discrepancies between the results of these two investigators appear confirmatory of our observations that the reduction of dyes, when in mixtures with organic complexes such as milk, does not always take place over a potential range identical with the theoretical value for the dye.

Quastel and Wooldridge (1927) advanced the theory that

the dehydrogenations effected by bacteria are primarily due to polarisations of substrate molecules induced by electric fields which characterise particular centres—the "active centres"—of cellular and intracellular surfaces. . . . . . The hypothesis we put forward, that enzymic activity may be regarded as the property of the active centers of cellular and intracellular structures (and this includes the smaller structures capable of extraction from or secretion by the cell) leads to a considerable simplification of the above view. [Note: These authors refer to a popular conception of enzyme behaviour which they had just discussed.] Precisely what enzymic behaviour a particular structure or colloidal aggregate in the cell may possess depends on the nature of the active centres which form a part of the structures or of the colloidal aggregates. . . . . Thus, we may imagine that the protein, nucleotides, etc., are not only so arranged as to form the various substances of the cell but that the arrangement is such that the active centers are formed on these particular substances. Enzymes, therefore, and cellular structures are inseparably connected. . . . . We may regard the entire aggregate as the enzyme, or the particular center as the enzyme.

This conception, though a step in advance of older enzymic concepts, seems inadequate as an explanation of the reduction of methylene blue in solutions containing no cells and no colloidal surfaces, as for instance in sugar solutions, unless we consider the sugar molecules to be in aggregates large enought to provide "active centers" on their surface but small enough to be in true solution. These authors do not report in this paper the potentials of their solutions or the poising effect of methylene blue.

It is impossible, therefore, for a reader to comment on the soundness of some of their conclusions.

Enzymes are believed to be found only where protoplasm exists or has existed. The constituents of the protoplasm of microörganisms are necessarily derived from media. Some of the media have been shown to have reducing properties. The indications are that these reducing properties are imparted to the media by simple and definite chemical constituents. It is reasonable to attribute protoplasmic reductions to chemical compounds as simple and definite as those found in bacteriological substrates. The characteristics usually ascribed to enzymes are met in the common media. If the reduction of dyes in milk is carried on by enzymes, we are at a loss to know their exact function. In the light of present knowledge it is improbable that they are hydrogen donators unless we conceive of them as identical with metabolites. Nor is it any more probable that they act as oxygen acceptors. Barthel's experiments with "synthetic milk" suggest that the milk salts act as catalyzers. Enzymes would, therefore, seem to be superfluous for this purpose. If colloid surfaces are necessary for reduction centers or points, these are already provided by the natural colloids of the milk. Certain it is that, if reduction of dyes in milk is due to enzymes, we must of necessity revise our conception of the heat lability of reductases.

The rôle of bacteria

The relationship of bacteria to methylene blue reduction times in milk has received such an enormous amount of attention that there can be, now, no question of a quantitative relationship. Nevertheless, the function of the bacteria in the reduction test is still a controversial matter. It has been believed by many that reduction of the dye is caused by the bacteria directly. If this is true, then the point of reduction is still undetermined. There seem to be four possibilities in this regard, viz.: (1) Within the bacterial cell, (2) At the surface of the cell, (3) At a distance from the cell due to diffusible substances elaborated by the cell, presumably enzymes, and (4) a combination of any two or all of these.

As we have already pointed out, it is probable that all protoplasm has a reducing power. Needham and Needham (1926), Cohen, Chambers, and Reznikoff (1928), and others have demonstrated such power in the protoplasm of amoeba and other cells. It is reasonable to ascribe a similar property to the protoplasm of the bacterial cell. But it is highly improbable that the major reduction of the dye in the methylene blue reduction test takes place within the bacterial cell or at the surface of the cell. Plate counts of 38 samples of milk taken at the moment of reduction varied from 3.5 million to 45 million with an average count of 21 million bacterial colonies per cubic centimeter. The microscopic picture obtained with a stained smear of such milk shows uneven distribution of the bacteria and is not one which would lead to an expectancy of even disappearance of the dye from the milk, if all reduction were taking place within the cell or at the surface of the cell. In other words, 21 million points of reduction per cubic centimeter seem too few to allow for a conception of homogeneous distribution and disappearance of the dye. We are unable to conceive of a mechanism which would allow such a rapid return of the blue color to the milk on shaking a reduced sample with air if the oxidative process must take place within, or at the surface of, the cell.

Reduction of methylene blue in milk by bacteria has been reported in concentrations as high as 1:3000. In 1 cc. of such milk there would be 0.00033 gram of dye. If we assume that 100 million bacterial cells are present in each cubic centimeter of the milk at the time of reduction and that 100 million bacteria weigh 0.0001 gram, then a much greater weight of dye than of bacteria would be present. It is inconceivable that this amount of dye would concentrate in the living cells. We have already shown that the reducing properties of raw milk are sufficient to account for the reduction of the dye without the aid of bacteria.

All of our work tends to confirm the hypothesis of Barthel (1917) that the disappearance of methylene blue in raw milk takes place in two stages, viz.: (1) the removal of the dissolved oxygen by bacteria; (2) the reduction of the dye by constituents of the milk. It is now amply demonstrated that the milk itself

assists the bacteria in fixing the free oxygen, but this process is so slow as to be of no great importance in the reduction test as ordinarily employed.

TABLE 4

The effect of varying concentrations of dye upon the reduction times of seven samples of milk

CONCENTRA-TION	TUBE NUMBER	MILK SAMPLES						
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
1:400,000	1			0:45	4:15	6:50		
	2			0:45	4:15	6:50		
1:330,000	1			0:45	4:15	7:15	4:00	
	2			0:45	4:20	7:15	4:00	
1:280,000	1			0:45	4:25	7:25	4:00	
	2			0:45	4:35	7:30	4:00	
1:250,000	1	1:05	2:55	0:45	4:35	7:20	4:15	
	2	1:05	3:05	0:45	4:35	7:50	4:15	
1:220,000	1	1:00	3:00	0:45	4:35	8:00	4:30	
	2	1:00	2:55	0:45	4:35	8:00	4:30	
1:200,000	1	1:05	3:00	0:45	4:35	8:05	Broken	4:30
	2	1:05	3:00	0:45	4:35	8:00	4:40	4:45
1:180,000	1	1:00	3:12	0:45	4:35	8:20	4:50	
	2	1:00	3:00	0:45	4:35	8:05	4:45	
1:160,000	1	1:05	3:00	0:45	4:45	8:35	4:50	4:35
	2	1:07	3:05	0:45	4:45	8:45	4:50	4:45
1:150,000	1			0:45	4:45	8:40	4:50	
	2			0:45	4:50	8:20	4:50	
1:140,000	1			0:45	4:45			4:35
	2			0:45	4:50			4:55
1:120,000	1							4:45
	2							4:55

THE REDUCTION CAPACITY OF MILK

Clark distinguishes between reduction "capacity" and reduction "intensity." The reduction "capacity" of milk is, loosely,

the amount of dye which the milk will reduce when the potential or "intensity" is such that reduction is possible.   The standard concentration of methylene blue used in the reduction test is 1 part of dye to 200,000 parts of milk.   This concentration has been found to impart a sufficiently deep blue color to the milk to give a comparatively sharp endpoint.   The lower the concentration of the dye the less will be the inhibition of bacterial growth.   If the reducing capacity of milk is sufficiently large to allow the reduction of dye in a 1:100,000 concentration in the same time as a 1:200,000 concentration, then accurate measurements of the milk samples, within these limits, is not of great importance.   Small errors in measurements necessarily occur in

TABLE 5

Reduction times of raw milk with varying dye concentrations, the oxygen being removed by hydrogen or carbon dioxide

DYE CONCENTRATION	REDUCTION TIME
1:200,000	0:30
1:100,000	0:40
1:40,000	2:07
1:20,000	Not reduced in 3 hours
1:10,000	Not reduced in 3 hours

practice.   A series of experiments was, therefore, undertaken to determine the effect of such errors.   The results of experiments with seven samples of milk to which the dye was added in varying concentrations are given in table 4.

These data show that there may be a slight increase in the reduction time with increase in the concentration of the methylene blue.   The concentration must be approximately doubled before any appreciable increase in reduction time is observed. To see if this increase of reduction time is due to the limiting "capacity" of the milk or to an increasing antiseptic effect, a series of tubes containing 10 cc. of fresh morning herd milk of low bacterial content were treated with varying concentrations of methylene blue.   They were stoppered but connected by means of glass tubing.   After being placed in the water bath washed

molecular hydrogen was bubbled through them in the order in which they are reported in table 5.

Tubes of the same milk were subjected to a similar manipulation after being heated in the autoclave at 15 pounds pressure for one hour. In this case the washed gas was led first through the milk of higher dye concentration, then through the milk of lower dye concentration. Identical results were obtained with the same milks when carbon dioxide was substituted for hydrogen. The results with the heated milk are given in table 6. These data indicate that any considerable increase in the reduction time of a dye concentration of 1:100,000 over that of a concentration of 1:200,000 is due to the antiseptic effect of the dye. This conclusion is in accord with that of Hastings, Davenport, and Wright (1922).

TABLE 6

Reduction times of heated milk with varying dye concentrations, the oxygen being removed by hydrogen or carbon dioxide

DYE CONCENTRATION	REDUCTION TIME
1:100,000	0:20
1:200,000	0:10
1:200,000	0:10

Another sample of milk was autoclaved with varying dye concentrations and 1 part of dye in 1000 parts of milk was reduced, while no reduction was observed when the dye concentration was 1:100. This does not necessarily limit "capacity" to this figure; it is possible that the "poising" action of the methylene blue in these high concentrations is great enough to prevent the falling of the potential through the range of the dye. Whether heating milk increases the reducing "capacity" cannot be said. Dubos (1929) failed to take "poising" action into consideration in attempting to measure the reducing capacity of nutrient broth. His data suggest the influence of this factor. It is evident from these figures, however, that the reducing "capacity" of raw milk is sufficient to take care of double the dye concentration usually employed in the test.

THE RATE OF REDUCTION

Attempts have been made to measure the rate of reduction of methylene blue in milk by titration with titanium trichloride. The investigators using this method have concluded that reduction of the methylene blue begins immediately on addition of the dye to the milk.   This is contrary to the common observation that the disappearance of the blue color is usually rapid when once decolorization sets in.   In many milks, even in long-time reducing milks decolorizing evenly, the intense blue color remains to within five, ten, or fifteen minutes of complete visual reduction. The reoxidation of the dye on shaking with oxygen is relatively

TABLE 7

Comparison of reduction times when methylene blue was added at the start and near completion of the test

DYE ADDED AT START		DYE ADDED AFTER 12 HOURS	
Number of tubes	Reduction time	Number of tubes	Reduction time
1	12:30	7	12:30
2	12:45	6	12:45
1	13:00	2	13:15
2	13:15	1	13:30
Average	12:55		12:46
Variation	0:45		1:00

rapid.   Our experiments with deaerated milks substantiate the theory that the reduction of the dye in the standard concentration is also rapid.

Approaching the problem in a different way, a long-time reducing milk was chosen.   To six tubes of this milk the usual dye concentration was added at once and these tubes were then placed in the water bath with sixteen tubes of the same milk to which the dye was added after twelve hours incubation.   The results are given in table 7.   As will be seen from the table, complete visual reduction was effected in 7 of these tubes within thirty minutes of the time the dye was added.   Variation of reduction times in duplicate tubes of milk and uneven disappearance of the dye in the same tube will be given full consideration in a later paper of this series and a theory offered in explanation.

If the reduction of methylene blue in milk is effected by enzymes elaborated by the bacteria, which was the popular theory up to very recent times, it seems necessary to assume that reduction of the dye begins immediately on addition to the milk. The experimental results tabulated above are explainable on the older basis if a concentration of enzymes is built up in the milk. Nevertheless, the question is pertinent, is titanium trichloride specific for methylene blue? The titration curves reported by Fred (1912) are remarkably similar to the potential-time curves obtained by Clark and by ourselves. This suggests that oxygen was being measured. It has been adequately demonstrated that the older hypothesis of dye reduction is untenable. Clark (1925) reports the potential (E_h) ranges within which methylene blue changes from 4 per cent reduced to 96 per cent reduced at different hydrion concentrations. Complete visual reduction is within a narrower range than this. The rate at which methylene blue will reduce in milk depends upon the speed with which the potential ("intensity") passes through this range. Examination of the charts in the cited paper and those reported in this paper will leave no doubt that the time taken for the potential to pass through the range of the methylene blue-methylene white reaction is usually short.

SUMMARY

Potential-time curves of milk and milk methylene blue mixtures are given. The positive E_h limits of all the milks lay between $+0.2$ and $+0.3$ volt. The negative potential limits reached by all the milks reported in this paper approximated E_h -0.2 volt, due, it is suggested, to the predominating influence of *Streptococcus lactis*.

The range of reduction of methylene blue in milk has been observed to be more positive than the theoretical value at this pH, and varies considerably in different milks.

An explanation is suggested for the upward trend of the potential curve sometimes noted after the negative limit is reached.

The "poising" effect of methylene blue in the standard concentration in milk is so small as to be almost negligible.

The rôle of hydrogen in reduction is discussed, and the effect of some substances upon reduction times shown, as well as the effect of cysteine upon potentials in milk.

The rôle of oxygen is discussed and evidence presented of its relation to reduction and to reduction potentials.

The necessity for revision of enzyme concepts is made apparent and attention is drawn to the need for consideration of the reducing properties of bacteriological media.

All of our work tends to confirm the theory of Barthel that the disappearance of methylene blue in the reduction test in milk takes place in two stages, viz.: (1) the removal of the dissolved oxygen by bacteria; (2) the reduction of the dye by constituents of the milk.

Evidence is brought forward to show that the reducing "capacity" of raw milk is sufficient to reduce a concentration of methylene blue of 1 part of dye to 100,000 parts of milk in approximately the same time as in the standard concentration, 1:200,000. The "capacity" is probably much greater than this. The "capacity" of heated milk is sufficient to reduce this dye in a concentration as high as 1:1000. We are not in a position to say that heated milk has a greater "capacity" than raw milk.

The rate of reduction of methylene blue in raw milk is rapid and depends upon the speed with which the potential passes through the range of this dye. The time taken for the reduction of the dye in normal raw milk is usually short in comparison with the time necessary for oxygen consumption.

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STUDIES ON OXIDATION-REDUCTION IN MILK

II. THE CHOICE OF AN INDICATOR FOR THE REDUCTION TEST. THE REDUCTION OF JANUS GREEN B IN MILK[1,2]

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In 1900 Neisser and Wechsberg suggested that the reduction of methylene blue might be used as an indication of the bacterial content of milk. The intervening twenty-nine years have witnessed the adoption of their suggestion and the growing use of their method till today it is one of the standard tests in the bacteriological analysis of milk. It is significant that methylene blue has remained undeposed as the indicator *par excellence* for this purpose. This dye has retained its place, not because of precise knowledge of its behavior in milk, but rather because its superiority has been amply demonstrated by experience and by trial and error methods of comparison. In the light of the newer knowledge of dye reduction the time seems ripe to reconsider the question of indicators for milk quality.

A splendid review of the history of dye reduction by bacteria is to be found in the paper by Fred (1912). A very extensive bibliography on methylene blue as an indicator of reduction has been given by Clark, Cohen and Gibbs (1925). This paper also discusses the place of methylene blue as an indicator in biological oxidations and reductions. A reiteration here of material so readily available would be out of place. The reader is, therefore, referred to the cited sources for such information.

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[1] Published with the permission of the Director of the Agricultural Experiment Station.

[2] The work on janus green was done by one of the authors (T.) at the North Dakota Agricultural Experiment Station, Fargo, N. D.

We are today in a position to postulate a few simple requirements to be met by an indicator for the purpose under discussion. Some of these are:

1. The range of reduction in milk must be negative to the potentials of fresh milk but should be only slightly so.
2. It should have a maximum tinctorial effect with a minimum antiseptic effect.
3. The color change should be sufficient to give a sharp end-point.
4. The poising effect in milk should be negligible.
5. It should be easily purified.
6. It should be stable in aqueous solution.

Let us now consider the comparative qualifications of methylene blue. Clark and his co-workers at the Hygienic Laboratory have shown that certain oxidation-reduction indicators have definite and reproducible potentials when in aqueous solution in equilibrium with their reduced forms. Cohen, Chambers, and Reznikoff (1928) list 24 such indicators with their E_h values at pH 7.0 when 50 per cent reduced. These dyes are visibly reduced within rather narrow E_h ranges having for their centers their E_h values when 50 per cent reduced. By observing progressive reduction of a number of such indicators in duplicate samples of milk the potential changes in the milk may be followed.

Among the dyes which we have studied in relation to the reduction test are, 2,6,dichlorophenol indophenol; ortho cresol, 2,6,dichloro indophenol; a naphthol sulphonate dichloro indophenol; toluylene blue; and potassium indigo tetrasulphonate; all supplied through the kindness of Drs. Clark and Cohen. We have also studied methylene blue, janus green B, litmus, and safranin. The first two indicators reduce almost immediately in fresh milk of low bacterial content. Their range of reduction is more positive than the potential values of such milk. Toluylene blue decomposed in milk at 37.5°C. The first three dyes have been found to be unstable on standing in aqueous solution in concentrations practical for this test. Methylene blue, on the other hand, gave identical results whether fresh or after standing in aqueous solution for some years protected from light. Potassium

indigo tetrasulphonate was not observed to have any advantages over methylene blue but has the disadvantage of reducing over a range more negative than that of methylene blue.   The same is true of janus green and litmus.   Safranin frequently does not reduce in milk, so negative is its range of reduction.

Ordinarily reduction of these dyes in milk will take place in the order named.   In one milk complete visual reduction of methylene blue was observed at E_h +0.1 volt, while visual reduction of the naphthol sulphonate dichloro indophenol indicator was not complete till E_h +0.075 volt was reached, although the reduction times were almost identical.   This dye should reduce at a more

TABLE 1

The reduction times of three dyes reducing over different potential zones in milk

MILK NUMBER	AN INDOPHENOL		METHYLENE BLUE	POTASSIUM INDIGO TETRA SULPHONATE	
	Reduction time	Difference	Reduction time	Reduction time	Difference
1	1:20	−1:05	2:25	2:30	+0:05
2	6:05	−0:20	6:25	6:35	+0:10
3	5:50	−0:45	6:35	6:35	0:00
4	0:15	0:00	0:15	0:40	+0:25
5	6:30	−1:15	7:45		

Note: The differences cited in this table are with reference to methylene blue reduction times.   2:25 means 2 hours and 25 minutes.

positive potential than methylene blue.   A difference in their antiseptic effect will have an influence on reduction time.   Of this effect little is known.

Table 1 gives the reduction times of a number of indicators in five milks.   It will be noted that reduction took place in most cases in the expected order.   In studying this table the following variables should be kept in mind:

1. The varying antiseptic effect of different dyes.
2. Marked variations of reduction times in duplicate tubes of the same milk.
3. The difficulty of accurately reading such a color test.

On scrutiny of this table it is observed that differences between methylene blue reduction times and those of the first dye are less regular than differences between methylene blue reduction times and those of the last indicator. Examination of figure 1 will disclose the explanation for this. The solid line is the potential-time curve of a long-time reducing milk. The broken-line curve represents the potentials in a duplicate sample containing methy-
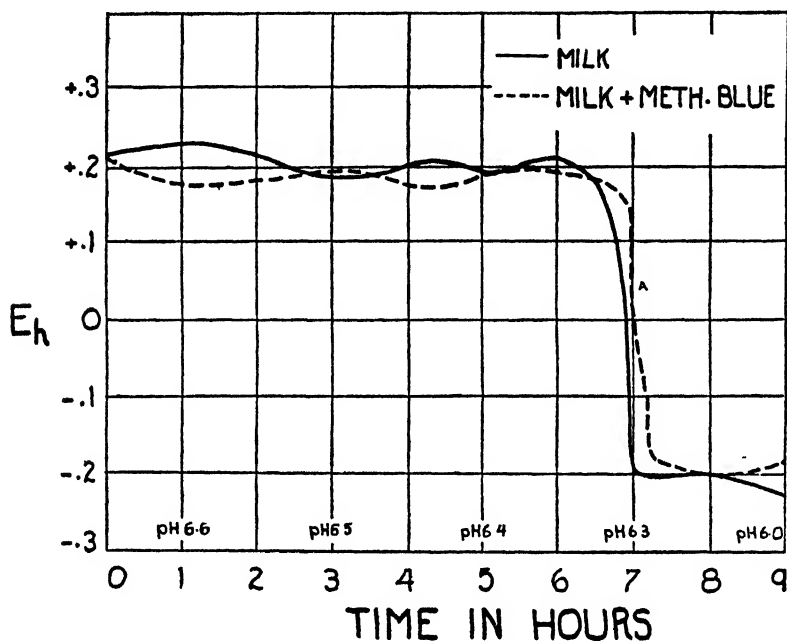
FIG. 1. POTENTIAL-TIME CURVES OF A SAMPLE OF MILK WITH AND WITHOUT THE ADDITION OF METHYLENE BLUE, SHOWING THE UNSTEADY POTENTIALS OBSERVED AT THE POSITIVE E_h LIMIT

lene blue which decolorized at point A. The unsteady potential illustrated in the upper portion of these curves is typical of such milks. The same phenomenon has been observed in yeast cell suspensions by Cannan, Cohen, and Clark (1926) who state that "electrodes which give potentials widely apart at the early intervals of time tend to approximate more and more, and, if time be allowed, will often come into excellent agreement." The differ-

ences between potential values as read with different electrodes appear to be no greater than the fluctuations with the same electrode during the time represented by the upper portion of the curve. The indophenol indicator used in the experiments reported in table 1 reduces over a potential range slightly positive to that of methylene blue. In the milk used for figure 1 complete visual reduction of the indophenol was observed at E_h +0.1 volt. The methylene blue was considered reduced at E_h +0.05 volt. These values are only approximate because of the rapid rate of fall of potential. The range over which the first indicator reduced was very close to the upper break in the curve. The potential fluctuation during the first six hours of incubation was great enough partially to reduce the dye and accounts for the irregular differences noted in table 1. It is apparent that the use of this dye might easily be misleading if reduction times are to be the criterion of quality. In no case have we observed the reduction of methylene blue at an E_h value close enough to the positive potential limit of fresh milk to make us suspect misleading results. The wish has been expressed for an indicator which would materially shorten the time required for the test. Our potential-time curves, in this and the preceeding paper, show the impossibility of such a thing.

Attention is again called to the close agreement of the positive potential limits of fresh milk reported by us with those reported by other investigators for milk and other biological fluids. The position of the range of reduction of methylene blue on the E_h scale in relation to the potential limits within which aerobic life seems to take place is significant. Without question, this characteristic of methylene blue is one of a number of reasons for the survival of this dye as an indicator in biological oxidations and reductions through years of elimination trials. This survival has been well discussed in the paper by Clark et al. (1925). These investigators report the difficulties encountered in attempts to purify methylene blue. This difficulty and the ease with which it adsorbs onto surfaces are the only objectionable features of methylene blue we have observed. So great is its affinity for surfaces that etched test-tubes are useless for the test. A faint

blue color frequently remains in the milk for some time after the main reduction has most apparently taken place. This may be due to impurities which reduce over a potential range negative to that of methylene blue. The high tinctorial properties of methylene blue are quickly recognized by any one working with various dyes. In the first paper of this series we have advanced evidence that the poising effect of methylene blue is negligible in milk and may be disregarded in the routine application of the reduction test. Of the indicators so far examined, it would seem that methylene blue is most suitable for this test.

Christiansen (1926) has strongly recommended the substitution of janus green for methylene blue in the reduction test. According to this worker the reduction of methylene blue, because of its reversibility, is retarded in the operation of the reduction test by the diffusion of atmospheric oxygen into the milk. He claims that janus green is not so affected, since the first reduction reaction of this indicator is considered irreversible. As evidence that methylene white is reoxidized again to methylene blue by atmospheric oxygen in the practical application of the test, Christiansen notes the constant disappearance of the color from the bottoms of the tubes first. It has been very striking in the milks with which we have worked that this phenomenon was not by any means invariably observed although it frequently was so. We have already (1927) advanced the theory that the sweeping of the bacteria out of the body of the milk by the rising of the butterfat and the precipitation of many cells to the bottom of the tube account for the uneven reduction of the dye. In no case were we able to observe a difference in the reduction times of whole milk attributable to the use of a vaseline seal.

Soep (1927) supports the Christiansen modification of the reduction test and reports that janus green has been substituted for methylene blue by many in Germany. He concludes that methylene blue reduces twice as rapidly as janus green in milks having reduction times of less than a half-hour: the differences in the reduction times of the two dyes become less as the reduction time increases, till finally methylene blue reduction times may be greater than those of janus green. He concurs with Christiansen in the opinion that diffusing oxygen is the cause.

TABLE 2

Methylene blue and janus green B reduction times in 157 samples of market milk

MILK NUMBER	METHYLENE BLUE	JANUS GREEN	DIFFERENCE	MILK NUMBER	METHYLENE BLUE	JANUS GREEN	DIFFERENCE
1	0:15	0:30	0:15	44	5:00	6:30	1:30
2	0:30	1:15	0:45	45	5:15	7:15	2:00
3	0:45	1:30	0:45	46	5:15	6:00	0:45
4	1:00	2:15	1:15	47	5:15	6:45	1:30
5	1:00	1:30	0:30	48	5:15	7:15	2:00
6	1:00	1:30	0:30	49	5:15	7:15	2:00
7	1:00	1:45	0:45	50	5:30	8:15	2:45
8	1:00	3:00	2:00	51	5:30	7:15	1:45
9	1:00	2:00	1:00	52	5:30	6:30	1:00
10	1:45	2:15	0:30	53	5:30	6:00	0:30
11	2:00	2:15	0:15	54	5:30	7:30	2:00
12	2:15	3:45	1:30	55	5:30	6:30	1:00
13	2:30	2:45	0:15	56	5:45	7:45	2:00
14	2:45	3:45	1:00	57	5:45	6:15	0:30
15	2:45	6:00	3:15	58	5:45	6:15	0:30
16	3:00	3:45	0:45	59	5:45	6:45	1:00
17	3:30	6:15	2:45	60	5:45	6:30	0:45
18	3:45	4:00	0:15	61	5:45	6:45	1:00
19	3:45	5:45	2:00	62	5:45	6:15	0:30
20	3:45	6:30	2:45	63	6:00	6:45	0:45
21	4:00	4:30	0:30	64	6:00	7:15	1:15
22	4:00	5:30	1:30	65	6:00	8:15	2:15
23	4:00	4:45	0:45	66	6:00	7:45	1:45
24	4:00	5:15	1:15	67	6:00	6:30	0:30
25	4:00	6:15	2:15	68	6:00	6:45	0:45
26	4:15	6:30	2:15	69	6:15	7:45	1:30
27	4:15	6:30	2:15	70	6:15	8:45	2:30
28	4:15	6:15	2:00	71	6:15	8:15	2:00
29	4:30	5:15	0:45	72	6:15	7:30	1:15
30	4:30	6:45	2:15	73	6:15	6:30	0:15
31	4:30	6:15	1:45	74	6:15	7:45	1:30
32	4:30	5:30	1:00	75	6:15	7:45	1:30
33	4:30	6:00	1:30	76	6:15	9:15	3:00
34	4:30	7:45	3:15	77	6:15	6:45	0:30
35	4:30	5:45	1:15	78	6:30	7:45	1:15
36	4:30	5:00	0:30	79	6:30	7:45	1:15
37	4:30	5:45	1:15	80	6:30	8:00	1:30
38	4:45	8:00	3:15	81	6:30	7:15	0:45
39	4:45	6:15	1:30	82	6:30	7:45	1:15
40	4:45	9:15	4:30	83	6:45	8:15	1:30
41	4:45	6:45	2:00	84	6:45	8:00	1:15
42	4:45	7:00	2:15	85	6:45	7:45	1:00
43	4:45	7:45	3:00	86	6:45	7:45	1:00

TABLE 2—*Concluded*

MILK NUMBER	METHYLENE BLUE	JANUS GREEN	DIFFERENCE	MILK NUMBER	METHYLENE BLUE	JANUS GREEN	DIFFERENCE
87	6:45	7:15	0:30	123	8:15	9:00	0:45
88	6:45	7:45	1:00	124	8:15	9:00	0:45
89	6:45	7:15	0:30	125	8:15	9:30	1:15
90	6:45	8:15	1:30	126	8:15	9:15	1:00
91	7:00	9:00	2:00	127	8:15	9:15	1:00
92	7:00	9:00	2:00	128	8:30	10:45	2:15
93	7:00	7:45	0:45	129	8:30	11:15	2:45
94	7:00	7:15	0:15	130	8:30	9:15	0:45
95	7:00	8:45	1:45	131	8:30	10:30	2:00
96	7:00	8:00	1:00	132	8:30	10:30	2:00
97	7:00	7:30	0:30	133	8:30	9:45	1:15
98	7:15	8:00	0:45	134	8:30	10:30	2:00
99	7:15	8:00	0:45	135	8:45	10:45	2:00
100	7:15	9:15	2:00	136	8:45	9:30	0:45
101	7:15	10:30	3:15	137	9:00	11:00	2:00
102	7:15	7:45	0:30	138	9:00	11:15	2:15
103	7:15	9:15	2:00	139	9:00	10:30	1:30
104	7:15	8:45	1:30	140	9:00	11:00	2:00
105	7:15	10:15	3:00	141	9:15	10:00	0:45
106	7:30	9:00	1:30	142	9:15	10:30	1:15
107	7:30	9:15	1:45	143	9:15	10:15	1:00
108	7:30	9:15	1:45	144	9:30	11:15	1:45
109	7:30	9:15	1:45	145	9:30	11:15	1:45
110	7:30	9:15	1:45	146	10:00	11:00	1:00
111	7:45	8:00	0:15	147	10:15	11:45	1:30
112	7:45	9:15	1:30	148	10:15	11:30	1:15
113	7:45	8:45	1:00	149	10:30	11:30	1:00
114	8:00	11:15	3:15	150	10:30	11:15	0:45
115	8:00	8:00	0:00	151	10:30	11:15	0:45
116	8:00	10:00	2:00	152	10:30	11:00	0:30
117	8:00	10:00	2:00	153	10:45	12:00	1:15
118	8:15	10:15	2:00	154	10:45	11:00	0:15
119	8:15	9:00	0:45	155	10:45	11:00	0:15
120	8:15	11:45	3:30	156	11:00	13:45	2:45
121	8:15	10:15	2:00	157	11:15	12:15	1:00
122	8:15	9:15	1:00				

In a study of the comparative reduction times of janus green B (Coleman and Bell) and methylene blue in 157 samples of milk from as widely separated sources as Fargo, North Dakota, and Calgary, Alberta, Canada, janus green B reduction times were longer than methylene blue reduction times in every sample but

one.   In one 8-hour milk the results were identical for both dyes.
Table 2 gives the reduction times of the two dyes in all the milks
while in figure 2 the average differences between their reduction
times are plotted against the average methylene blue reduction
times.   It is apparent that there was no tendency for the reduc-
tion times to be equal at six hours.   We wish to call attention to
the fact that reduction times in raw milk are dependent upon
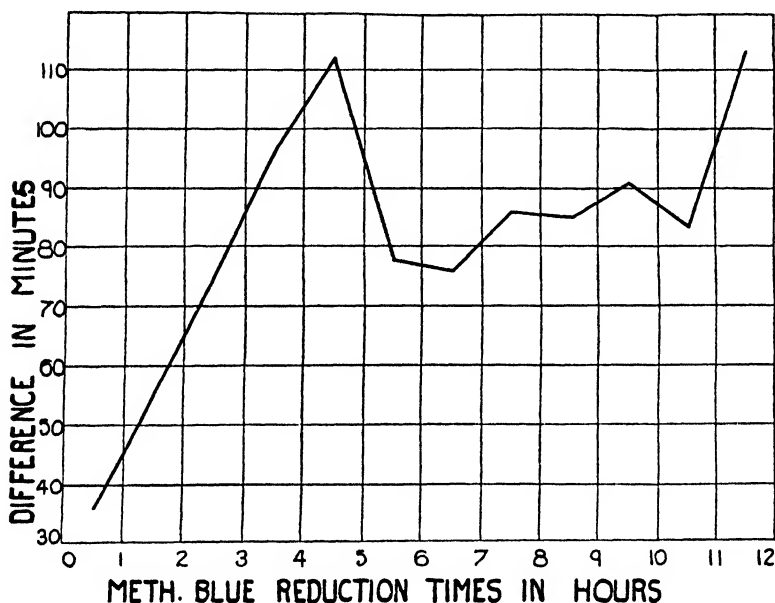
Fig. 2. Average Differences Between Methylene Blue and Janus Green B
Reduction Times in 157 Samples of Milk

bacterial growth which is not a linear function of time.   There-
fore, differences are not comparable on a percentage basis.   If
bacteria reproduce every half-hour during the test (this is proba-
bly approximately true), then a variation of a half-hour in reduc-
tion time means a variation of 100 per cent in bacterial numbers
whether reduction is effected in one hour or ten.

   A potentiometric study reveals the cause for the difference in
the reduction times of the two dyes.   In figure 3 the solid line is
the potential-time curve of methylene blue in its usual concentra-

tion in milk.   The broken line curve represents that of 1 part of
janus green B in 100,000 parts of the same milk.   This is the
concentration recommended by Christiansen.   At point *A* the
reduction of methylene blue had taken place.   At point *B* the
janus green B had reduced to the red compound.   It will be
observed that the latter dye reduces over a potential range more
negative than that of methylene blue.   This, in part, accounts
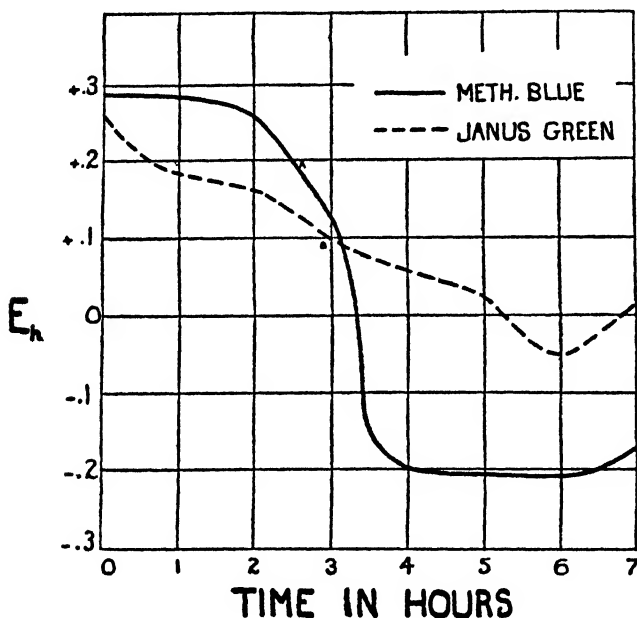
Fɪɢ. 3. Pᴏᴛᴇɴᴛɪᴀʟ-Tɪᴍᴇ Cᴜʀᴠᴇꜱ ᴏꜰ Jᴀɴᴜꜱ Gʀᴇᴇɴ B ᴀɴᴅ Mᴇᴛʜʏʟᴇɴᴇ
Bʟᴜᴇ ɪɴ Mɪʟᴋ

for the difference in the reduction times of the two dyes, and as
we have already pointed out, is not an advantage but probably a
disadvantage.

   In the first paper of this series curves are presented to show
that methylene blue in the standard concentration in milk has a
slight poising action, insignificant for all practical purposes.   As
shown in figure 3, janus green B in the concentration used in this
work has a strong poising action in milk.   Such a poising action

was observed in all but one of the milks which we examined poten-tiometrically. This, together with the more negative potential at which this dye reduces, explains the results reported with the 157 samples of milk. For the purpose of the reduction test, a marked poising action of a dye in milk is a disadvantage.

When 10 cc. of a whole milk methylene blue or litmus mixture was autoclaved in ordinary sized test-tubes, the rate of diffusion of atmospheric oxygen into the milk, as measured by the return of color to the dye, was found to be about 1 cm. in the first six hours and about 2 to 3 cm. in the first twenty-four hours. Add to this the effect of an aerobic flora, which doubtless plays an important part in the test as applied in practice, and it seems reasonable to conclude that the rate of diffusion of atmospheric oxygen into the milk is not sufficiently rapid to effect the test. This explains our results when reduction times were compared, with and without a vaseline seal.

The janus green B used in this work was di-methyl safranin linked through an azo group to di-methyl anilin. On reduction it is supposed that the azo linkage is first broken freeing a red compound, presumably safranin. This step is considered irrever-sible. A further reduction is possible and frequently takes place in raw milk, the red compound reducing reversibly to a leucobase. The first reaction is the one recommended as a substitute for the methylene blue methylene-white reaction.

Lewis and Lewis (1924) consider the reduction of janus green to be a reversible reaction in the technique of staining mitachon-dria. In a few samples of our milk a blue color similar to that of janus green B returned when the pink colored milk (reduced) was shaken with air. Nevertheless, in no case did the usual oxidizing agents, such as H_2O_2, cause a reappearance of the blue color. We are unable to find a theory of oxidation-reduction potentials which would explain a poising action with an irreversi-ble system. Yet the poising action of janus green B in milk is very noticeable. These observations have led us to wonder if a rearrangement of the molecule takes place giving a reversible system not unlike that of methylene blue. It has been suggested to us that an intermediate hydrazo compound might account for

the poising action of this dye. The latter seems probable in view of the work of Conant and Pratt (1926) who report that "the change of certain azo to hydrazo compounds can be shown to be reversible and the oxidation-reduction potential measured." This was shown to be so by Biilmann and Blom (1924). The potential range of reduction and the poising action of janus green B as shown in figure 3 may account for the behaviour of this dye in the staining of mitachondria.
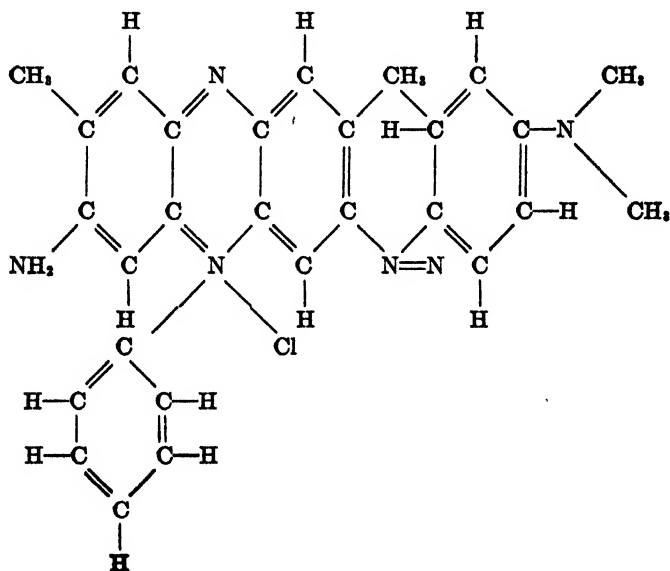
JANUS GREEN B

In the first paper of this series we have shown that the dissolved oxygen is the chief factor influencing the oxidation-reduction potentials in milk during the period of rapidly falling potential. Conant reports that the reduction of an irreversible dye takes place at a definite potential in a manner not dissimilar to the reduction of a reversible system. The potential of a system partially reducing the irreversible dye, he calls the "apparent" reduction potential. If the reduction of janus green B in milk is dependent upon the potential of the milk, as is the case with methylene blue and appears to be the case with janus green B,

then the effect of the diffusion of oxygen is the same for both dyes. This argument may be objected to because we have disregarded rates of reduction. If such a thing had an influence on our results we were unable to recognize it.

The end-point of reduction of janus green B in milk is much more indefinite than that of methylene blue reduction. This is due in part to the difficulty ensuing from the mixture of colors, i.e., the white of the milk, the blue of the janus green, and the red of the reduced compound. In part it is due to the poising action of the janus green B and the consequent lengthening of the time necessary for the potential to pass through the zone of reduction of this dye.

The comparative antiseptic effects of the two dyes are not known.

SUMMARY

There are given a few requirements which a dye must meet before it becomes useful as an indicator for the reduction test in milk.

Three indophenol indicators, toluylene blue, methylene blue, potassium indigo tetrasulphonate, janus green B, litmus, and safranin are compared as indicators for the reduction test in milk. The indophenols reduce over a potential range too positive for the purposes of this test. Toluylene blue decomposes in milk at 37.5°C. Potassium indigo tetrasulphonate, litmus, janus green B, and safranin reduce over a potential range more negative than is necessary for this test. This has the effect of lengthening the period of the test. So negative is the reduction range of safranin that milk does not always reduce it.

Methylene blue has a high tinctorial effect and sharp end-point; is stable in aqueous solution and in milk; has an inconsequential poising action in milk; and reduces over a potential range which particularly fits it for the reduction test. It has the disadvantage of being difficult to purify and adsorbs readily onto surfaces.

In 156 out of 157 samples of milk janus green B reduction times were longer than methylene blue reduction times. Potentiometric studies are reported which show that this is due to the more negative potential range of janus green B, as well as to its strong

poising action in milk. This difference in reduction times is shown to be independent of oxygen diffusion.

The end-point of reduction of janus green B is much less definite than that of methylene blue, due to the relatively strong poising action of the former dye and to the difficulty ensuing from a mixture of colors.

It is suggested that the poising action of janus green B is due to a rearrangement of the molecule or to the formation of an intermediate and reversibly reducing compound, probably a hydrazo compound.

There were observed no advantages of janus green B over methylene blue as an indicator of milk quality. On the other hand several disadvantages were found.

Of the nine indicators reported in this paper, methylene blue stands out preeminently as the best suited for the reduction test.

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THE RELATIVE THERMAL DEATH RATES OF YOUNG AND MATURE BACTERIAL CELLS

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The conception of a definite period of youth in the life of bacteria now appears to be well established.

From the standpoint of the initiation of reproduction in cultures it has long been known that transplants from cultures only a few hours old start reproduction at once, whereas inoculations from old laboratory cultures require a period of dormancy before reproduction begins. This phenomenon was observed microscopically by Barber (1908), and was later studied more in detail with the aid of growth curves by Penfold (1914) and Chesney (1916).

From the work of Clark and Ruehl (1919) and Henrici (1921) it is known that the morphology of bacterial cells varies greatly with the age of the culture. Henrici has carefully studied this subject and the biological significance of these morphologic changes has been interpreted by him in a series of subsequent papers (1928).

Sherman and Albus (1923) studied young and old bacterial cells with the idea of detecting physiological differences. A number of tests were found which revealed definite physiological distinctions between young and mature cells. Differences were observed with respect to acid agglutinability, and resistance to harmful environmental factors, such as extremes of temperature, germicidal substances and osmotic pressure. These workers concluded that a period of physiological youth is characteristic of bacterial cells.

Among the many problems with which the bacteriologist is confronted, none is of more importance than the factors which

influence the death rate of organisms at high temperatures. The purpose of this investigation was to obtain more definite information concerning the relative heat resistance of young and mature cells of a few important types of bacteria.

The cultures used in this study were six strains of *Streptococcus fecalis,* a relatively heat resistant species of streptococcus, and three strains each of two capsulated organisms which were isolated from "ropy milk."

Prior to the heat tests the cultures were grown in sterile skimmed milk for different periods of time in order to obtain cells of varying ages. The *Streptococcus fecalis* cultures were incubated at 37°C. before the heat treatment, while the slime producing organisms were incubated at laboratory temperature. A 1-cc. amount of the culture under test was transferred from the milk in which it was grown to a tube containing 9 cc. of sterile skimmed milk previously heated to the temperature (62.8°C.) used for these tests. The tubes in which the heat tests were made were closed with sterile rubber stoppers in order to prevent undue surface cooling by evaporation, and also to allow thorough mixing, by shaking, at the beginning of the heat treatment. At the end of the heat treatment, samples were obtained from near the bottom of the tube without agitation and without touching the inside surface of the tube above the heating medium. These precautions are essential in order to obviate experimental error due to the adhesion of the bacteria to the walls of the tube above the liquid.

The ordinary plate culture method was used for determining the bacterial counts before and after the heat treatment. The *Streptococcus fecalis* counts were made using lactose nutrient agar and the plates were incubated at 37°C. for two days. The capsulated organisms were plated on plain nutrient agar and the plates were incubated at room temperature and counted at the end of two days. As these capsule forming organisms grow rapidly at comparatively low temperatures and produce large colonies the two-day incubation period at laboratory temperature

was shown to be adequate. All plates were made in duplicate and each culture was tested a number of times, always obtaining similar results.

From the data reported in tables 1, 2 and 3 it may be seen that the thermal death rate of the young cells is distinctly greater

TABLE 1

The relative resistance to heat of bacterial cells of different ages
(Streptococcus fecalis)

CULTURE NUMBER	AGE	BACTERIAL PLATE COUNT		RATIO BEFORE:AFTER	PER CENT SURVIVAL
		Before pasteurisation	After pasteurisation		
	hours				
18	24	109,000,000	2,870,000	37:1	2.63
	48	135,500,000	2,150,000	63:1	1 58
	4	7,000,000	18,000	388:1	0 25
20	24	102,500,000	1,310,000	78:1	1.27
	48	106,500,000	845,000	126:1	0.79
	4	8,100,000	14,000	578:1	0.17
21	24	135,000,000	1,200,000	112:1	0.88
	48	110,500,000	1,445,000	76:1	1 37
	4	9,150,000	21,700	421:1	0.23
22	24	102,500,000	3,190,000	32:1	3 11
	48	113,000,000	1,905,000	58:1	1.68
	4	13,450,000	32,600	412:1	0.24
23	24	149,500,000	3,250,000	46:1	2.17
	48	121,500,000	445,000	273:1	0.36
	4	11,050,000	10,900	1013:1	0.09
24	24	87,500,000	470,000	186:1	0.53
	48	144,000,000	530,000	271:1	0 36
	4	12,000,000	2,400	5000:1	0.02

than that of the older cells. In addition some other points, perhaps, are worthy of being noted. In the case of *Streptococcus fecalis* incubated at 37°C., a temperature at which the growth rate is rapid and the whole growth cycle therefore of brief duration, the cells from the twenty-four-hour culture were apparently

more resistant than those from a culture forty-eight hours old.
This is entirely logical, as it might be expected that the cells

TABLE 2

The relative resistance to heat of bacterial cells of different ages

(Ropy milk organisms—Type I)

CULTURE NUMBER	AGE	BACTERIAL PLATE COUNT		RATIO BEFORE:AFTER	PER CENT SURVIVAL
		Before pasteurization	After pasteurization		
	hours				
	54	84,000,000	23,350	3,597:1	0.026
2	24	33,000,000	5,900	5,593:1	0.018
	6	17,850	<1	>17,850:1	<0.005
	54	91,000,000	45,000	2,022:1	0.0494
4	24	61,000,000	5,100	11,960:1	0.0083
	6	98,000	<1	>98,000:1	<0.0010
	54	136,000,000	93,500	1,454:1	0.068000
6	24	23,600,000	8,400	2,809:1	0.035000
	6	3,850,000	<1	>3,850,000:1	<0.000002

TABLE 3

The relative resistance to heat of bacterial cells of different ages

(Ropy milk organisms—Type II)

CULTURE NUMBER	AGE	BACTERIAL PLATE COUNT		RATIO BEFORE:AFTER	PER CENT SURVIVAL
		Before pasteurization	After pasteurization		
	hours				
	54	17,650,000	1,895	9,314:1	0.010700
12	24	1,190,000	<1	>1,190,000:1	<0.000084
	6	5,350,000	<1	>5,350,000:1	<0.000019
	54	21,900,000	6,750	3,244:1	0.030000
14	24	183,000	<1	>183,000:1	<0.000540
	6	485,000	<1	>485,000:1	<0.000206
	54	12,700,000	160	79,375:1	0.001200
16	24	1,680,000	<1	>1,680,000:1	<0.000060
	6	6,600,000	<1	>6,600,000:1	<0.000015

would lose some of their resistance before the population of the
culture begins its numerical decline. Of practical interest is

the fact that the thermal death rates of the young and old cells are so different that such commercially important organisms as the "ropy milk" bacteria may survive pasteurization or be entirely eliminated, depending upon the physiological age of the cells.

SUMMARY

Thermal death rates of young and mature cells of *Streptococcus fecalis* and of two species of capsulated bacteria were studied.

In each case the young cells were markedly less resistant than were the mature cells of the same organism.

It is thought that more emphasis should be placed on this physiological difference in practical work.

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ELECTROPHORETIC POTENTIAL AS AN AID IN IDEN-
TIFYING STRAINS OF THE B. COLI GROUP

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The object of this paper is to show that the electrophoretic potential (P.D.) can be accurately determined for a given strain of the *B. coli* group by a simple technic and that the P.D., as determined by this method, is constant for that strain. It follows from this that, since strains differ considerably in their potentials, the P.D. can be used to assist in demonstrating the similarity or dissimilarity of two cultures of the coli group in a manner similar to the use of blood grouping in relation to paternity. In most of our strains, dissociants have given similar potentials but we have observed a few strains, the dissociants of which have given different potentials, each one being constant for its dissociant. As we have shown in another paper, this potential bears a direct relationship to the virulence of *B. coli*.

The most convenient apparatus for this work is the capillary cell type described by Falk (1928). The vital element of this cell is the capillary tube. The bore of commercial tubes varies considerably in spite of advertised tolerances. However, the bore is secondary in importance to the cleanliness of the tubes. All our tubes are freed from moisture by suction, filled with dichromate-sulfuric cleaning fluid, boiled in this fluid and washed with fat-free distilled water. They are then dried with a current of air.

Since the P.D. is influenced by differences in hydrogen-ion concentration, the presence of various ions, previous washing of the bacteria, the age of the culture, dehydration of the culture medium and some other factors, it was essential to study these variables and to formulate a standard technic in order that the

results obtained in one laboratory could be comparable with those from any other.

Our first problem was to control the pH of the growth. We attempted to do this by means of the usual amounts of phosphate buffer salts but found that they were hopelessly inadequate. When, however, the phosphate content was increased to 15 grams of each per liter, there was only a slight change in reaction in four hours. If the cultures were incubated for a longer period, the results became inconsistent and wide differences in pH were often observed in five to six hours. Since there was enough growth in three hours to make 1 or 2 cc. of suspension, and as the amount of metabolic by-products in this short incubation period would be slight, rendering washing unnecessary, we decided upon this as the optimum growth period. The medium finally adopted had the following composition:

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Agar........  . ....   . .  ..      . ......      ...... ...   15 grams
Peptone...............  . .   .   .............  ....  .   ...  10 grams
Meat extract  ....    ..      .....   ..  .. .....  .          3 grams
Mono sodium phosphate..    .. ...  .. ... ...  .....   ..     15 grams
Di-sodium phosphate   .      ... .. ....     .... ..         15 grams
Bromthymol blue..     .  .    ....  ..     .     . . . . 0 08 grams
Water to make.......   . .          ....  . .  .. .... 1000 cc.
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The reaction after sterilization is pH 6.75.

After three hours incubation on this medium, the growth is suspended in fat-free distilled water and diluted to a concentration of about 500 millions per cc. as judged by opacity. A capillary tube is filled immediately with the suspension and put in a Falk cell which has been connected to an E.M.F. of 45 volts. The speed is determined, preferably, by means of the new Jaquet precision stopwatch. Five determinations are made in one direction and the polarity of the cell is then reversed. Another five determinations are made which should compare favorably with the first set. The tube is then replaced by a second capillary filled from the suspension and the speed determined as with the first one. The two sets of determinations should agree within 4 per cent. In case of a disagreement, it is necessary to repeat the tests with other capillaries or to make fresh suspensions.

When reasonable checks have been obtained, it is desirable to convert the observed speeds into standard units. We prefer to use microns per 1000 volts per second since, with this method, the P.D. falls between 25 and 100. This is also convenient since it is sometimes desirable to use other than 45 volts. For rapid calculation, tables can be prepared for converting the observed speeds into standard units.

Having rigidly controlled the technic, it was next important to show that the P.D. is constant under these conditions and that it is constant for a given strain over a period of time. Two strains having quite dissimilar indices were tested a number of times on different plates of media and the P.D. was found to be contant for each. They were then plated daily for a month with very consistent results. Finally, they were put into daily use over a period of six months and the results obtained at the end of each two weeks were as follows:

    Strain 1..............48, 47, 48, 50, 50, 49, 48, 48, 47, 48, 48, 50 Av. 48
    Strain 2..............87, 91, 82, 92, 87, 89, 86, 90, 83, 83, 87, 90 Av. 87

Considering differences that might exist in culture media and in other experimental conditions, these results are quite constant. Some of the variations which did appear were found to be due to storage of the culture medium.

When these two standard strains are run on the same plates as unknown strains, we obtain rigid checks on the condition of the culture medium and the resulting growth. If the two standard strains do not give the correct P.D. that has been found from the average of a large number of determinations, it is evident that the unknown strains will also give unreliable results. In such instances, the tests are always repeated until satisfactory results are obtained. With these safeguards, we believe that the P.D. of the *B. coli* group can be accurately measured. In order to assist other laboratories, we will gladly send cultures of our two standard strains on request. Transplants are being offered to the American Type Culture Collection to perpetuate this standardization.

We have used this method to identify a given strain that has

been repeatedly isolated from the same person's stools; to investigate the possibility of the same strain existing in the stools of various members of a family; to determine if a given strain of *B. coli* has been successfully implanted in the intestinal tract; to determine if several colonies are identical, as, for instance, in the isolation of bacteria from water or feces; and to assist in identifying strains of the same organism isolated on different culture media.

These studies have been confined to the group of Gram-negative intestinal bacilli since their conditions of growth are admirably adapted to the above technic, but it is possible that, with modifications, the method may be of value in other groups of bacteria.

SUMMARY

1. A standard technic has been described for the determination of the electrophoretic potential (P.D.) of the coli group.

2. This potential is shown to be constant for each strain.

3. The value of the P.D. as an aid in comparing strains of the coli group is pointed out.

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STUDIES ON LEPTOSPIRAE

I. SOME OBSERVATIONS ON THE DISTRIBUTION AND CULTIVATION OF LEPTOSPIRAE

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INTRODUCTION

Spirochaetes of the genus now recognized as Leptospira were first described in 1914 by Wolbach and Binger (1914) who found them in water from fresh-water ponds. Similar organisms have been described since by Hindle (1925) from London tap water, by Bach (1921) from the drinking water in Bonn, by Noguchi (1922) from water samples collected in Massachusetts and New York, by Zuelzer (1923) in the tap water of Berlin, by Walker (1927) from the tap water of Washington, D. C., by Dimitroff (1927) from numerous sources of drinking water supplies in Massachusetts, Rhode Island and Connecticut and by Sardjito and Zuelzer (1929) in East Sumatra.

Besides these reports, which, by the way, indicate the wide distribution of these organisms in areas where infectious jaundice does not occur, there have been numerous observations of similar spirochaetes in the water in localities where this disease has not been uncommon.

It is not our purpose, however, to emphasize the coincident occurrence of leptospirae and Weil's disease but rather to call attention to certain observations we have made with regard to the distribution of these organisms in the water around Baltimore and to point out certain tentative conclusions which seem to be indicated from our cultivation experiments in the laboratory.

The water samples cultured for leptospirae in the course of this investigation came from a variety of sources. Some were taken from the tap in the laboratory, others were taken from the city water supply at various steps in the purification process, others were collected from aggregations of water in two of the city dumps, others from standing and flowing water in rural districts, others from springs and wells, and still others from the taps and the water filtration plants in Washington, D. C.

In all, 47 sources were examined for the presence of leptospirae. Some of the sources were repeatedly cultured, the period of sampling extending over the autumn, winter and spring months. In other instances only one or two samples were obtained from a given source. In this way information as to the multiplication, or at least the survival, of these organisms under all sorts of temperature variations was obtained. Furthermore, many of the samples were replated several times after varying periods of storage in the laboratory and data thus secured as to the longevity of these forms under storage conditions.

TECHNIQUE

In somewhat similar examinations Dimitroff (1927) grew leptospirae from water in petri dishes containing 1 to 2 per cent of sterile tap water fecal emulsion as the nutritive medium. To this, about 100 cc. of the water from each sample were added and the cultures incubated at room temperature in the dark and examined under dark-field illumination weekly for five weeks. The organisms found in his investigation multiplied scantily at 31° to 35°C., and the optimum temperature for their growth was said to be 25° to 28°C.

In Walker's (1927) experiments on the cultivation of leptospirae from water the nutritive material in the culture medium was at first likewise furnished by a portion of feces following the suggestion of Hindle (1925). However, it was soon found that other materials, such as hay infusion, broth, milk, egg, serum and blood could be substituted for the feces, and an egg-yolk semisolid agar medium could be used. To make this medium, 1.0

gram of agar was dissolved in 300 cc. of hot sterile tap water and the solution then cooled to 46°C. At this point 1.0 cc. of egg yolk was added aseptically, the mixture was thoroughly stirred together and held at 46°C. to prevent solidification of the agar until used.

In this investigation, when it was desired to make plates, 10 cc. of the water sample were placed in each petri dish and 20 cc. of the above medium added. In the examination of each water sample 4 plates were made; two were kept at room temperature and two were incubated at 37°C.

In what we termed our preliminary work, which consisted of the examination of 30 water samples, it was found that plates inoculated as indicated above might be positive—that is, show active leptospirae under the dark field—in from 5 to 15 days. Consequently, thereafter, the first examination was made after 14 days incubation and repeat examinations made after 3 and 4 weeks incubation. This applied to both the negative and positive plates and incidentally it may be said that whenever a plate was found positive on the first examination it remained so until it dried up.

THE OCCURRENCE OF LEPTOSPIRAE IN WATER FROM 47 SOURCES

Table 1 gives the details as to the types of sources, the period during which samples from the various sources were examined, the total number of samples examined from each source and the results of cultivation in the laboratory in terms of the number of samples from each source in which leptospirae grew out in the culture medium at either room or body temperature.

Inspection of table 1 indicates certain tentative conclusions. In the first place, it appears that leptospirae are not restricted in their distribution in natural waters as they grew in abundance in our plates inoculated with water from all sorts of sources that one might expect would be subject to contamination by wild rats acting as carriers of leptospirae. Such samples were gathered from standing water in the city dumps. Other samples, taken from sources open to possible fecal contamination by man or domestic animals, yielded leptospirae. Samples taken from

TABLE 1

Indicating the occurrence of leptospirae in water from 47 sources incubated at room temperature and at 37°C.

SOURCE	TIME OF SAMPLING	NUMBER OF SAMPLES EXAMINED	NUMBER OF SAMPLES POSITIVE	
			At 37°C.	At 20° to 25°C.
Brook running through meadow and receiving drainage from duck pond and cow pasture. Clear water	November 11, 1928, to April 8, 1929	6	0	2
Running stream. Drainage through tilled land and woodland. Clear water	November 11, 1928, to April 8, 1929	6	0	4
Gunpowder River	November 11, 1928, to April 8, 1929	6	5	4
Stream draining pasture	November 11, 1928, to April 8, 1929	6	1	5
Chinquepin Run. Decaying leaves and organic material	October 5, 1928, to April 15, 1929	7	5	6
Chinquepin Run. Drains area of woodland. ¼ mile from land that is pastured	October 5, 1928, to April 15, 1929	7	5	6
Stream draining entirely wooded area	October 5, 1928, to April 15, 1929	7	3	7
Stream flowing through meadow. Certain amount of pollution from houses probable	October 5, 1928, to April 15, 1929	7	5	7
Stream draining cow pasture, filled with grass, leaves, etc.	November 22, 1928, to April 15, 1929	6	2	6
Running water in Clifton Park heavily polluted with kitchen wastes	December 16, 1928	1	1	1
Stagnant water at dump in Clifton Park	December 16, 1928	1	1	1
Running water at dump in Clifton Park. Stream filled with rusty cans, etc.	December 16, 1928	1	1	1
Standing water at dump on Echodale Avenue. Numerous rats in evidence	December 16, 1928	1	1	1
Running water at dump on Echodale Avenue. Numerous rats in evidence	December 16, 1928	1	1	1

TABLE 1—*Continued*

SOURCE	TIME OF SAMPLING	NUMBER OF SAMPLES EXAMINED	NUMBER OF SAMPLES POSITIVE	
			At 37°C.	At 20° to 25°C.
Spring at Fruit Farm	November 11, 1928, to May 1, 1929	7	0	0
Spring at base of a hill used for drinking purposes. Open to surface wash	November 25, 1928, to May 1, 1929	6	0	0
Spring (Taylor Avenue)	April 21, 1929, to May 8, 1929	2	0	0
Store pump. Well 30 or 40 feet deep	May 1, 1929	1	0	0
Spring. Flows continuously through pipe. Green scum in pipe	May 1, 1929	1	0	0
Farm. Pipe leading from reservoir fed by spring. Gravity flow	May 1, 1929	1	0	0
Store pump from well 40 feet deep	May 1, 1929	1	0	0
Spring. Sample taken from concrete reservoir in spring house	May 1, 1929	1	0	0
Pump. Sample from well 40 feet deep. Open to surface contamination	May 1, 1929	1	0	1
Spring	May 1, 1929	1	0	0
Run-off from spring	May 1, 1929	1	0	0
Faucet	May 1, 1929	1	0	1
Chevy Chase tap water	November 17, 1929	1	1	1
Montebello Filtration Plant. Raw water at inlet	December 6, 1928, to May 7, 1929	5	1	3
Montebello Filtration Plant. Settling tank after addition of alum	December 6, 1928, to March 23, 1929	2	0	1
Montebello Filtration Plant. Wash water from filter bed	December 6, 1928	1	1	1
Montebello Filtration Plant. Water after filtration	December 6, 1928	1	0	1
Montebello Filtration Plant. Outlet 50 yards from point of chlorination	December 6, 1928	1	0	0
Montebello Filtration Plant. Settling tank after addition of alum and chlorine	February 2, 1929, to May 7, 1929	3	0	0

TABLE 1—*Concluded*

SOURCE	TIME OF SAMPLING	NUMBER OF SAMPLES EXAMINED	NUMBER OF SAMPLES POSITIVE	
			At 37°C.	At 20° to 25°C.
Montebello Filtration Plant. Wash water from filter bed. Water pre-chlorinated	February 2, 1929, to March 22, 1929	2	0	0
Montebello Filtration Plant. After filtration. Alum and chlorine added before filtration	March 22, 1929, to May 7, 1929	4	0	0
Montebello Filtration Plant. Outfall to city pipe line	March 22, 1929, to May 7, 1929	4	0	0
Baltimore tap water—supplied by Montebello Filtration Plant	October 17, 1928, to May 7, 1929	9	4	6
Washington Water Supply. Raw water	May 7, 1929	1	1	1
Washington Water Supply. Treated with alum 8 mi. away. Sample taken at old plant before filtration	May 7, 1929	1	0	1
Washington Water Supply. Filtered water at old plant	May 7, 1929	1	0	0
Washington Water Supply. Filtered water treated with lime at old plant	May 7, 1929	1	0	0
Washington tap water supplied by old plant. Water is chlorinated as it enters the main	October 23, 1928, to May 7, 1929	3	0	3
Washington Water Supply. Dalecarlia plant. Settled water prior to filtration. Coagulated and settled at plant	May 7, 1929	1	1	1
Washington Water Supply. Dalecarlia plant. Filtered water	May 7, 1929	1	0	0
Washington Water Supply. Dalecarlia plant. After chlorination but before addition of lime	May 7, 1929	1	0	0
Washington Water Supply. Dalecarlia plant. Chlorinated and limed. Storage reservoir	May 7, 1929	1	0	0
Washington tap water—supplied by Dalecarlia plant	May 7, 1929	1	0	0

brooks draining duck yards, cow pastures or obviously receiving domestic wastes fall into this category. On the other hand samples taken from certain streams draining woodland and other uncultivated areas where the opportunity for fecal contamination was slight were equally good sources of these organisms. In fact it was found possible to cultivate leptospirae from all kinds of natural waters, whether they were apparently open to contamination or not. An exception to this statement must be made in the case of some of the springs cultured. Without exception these samples were negative and there is no obvious explanation for this fact. Of this we are certain, however: it is not correlated with an absence of natural fauna as frogs were present, nor is it

TABLE 2

Comparison of results obtained from cultivation of spring and surface waters

SOURCE	TOTAL NUMBER OF SAMPLES	NUMBER OF POSITIVE SAMPLES	NUMBER OF NEGATIVE SAMPLES
Spring water..................	24	1*	23
Surface water..................... . ..	69	57	12

* Sample taken from well open to heavy surface contamination.

related to abundance of rotting grass and leaves along the margin of the spring. Searching further for an explanation of this phenomenon and bearing in mind Sardjito and Zuelzer's (1929) observation that leptospirae were absent in acid waters we tested the samples from the springs and found that they varied insignificantly from neutrality. It thus appears that the fact stands as an observation without adequate explanation, although it must be admitted that a more extensive examination of such sources would perhaps yield a different result.

Results identical with those obtained in the case of the springs were obtained from samples taken from deep, i.e., driven wells, although only two such sources were investigated. Neither of these wells yielded leptospirae in our cultures. The third, which did, was a dug well, open to obvious surface contamination from water running back into it through the wooden well cover.

A summary of the results obtained from cultivation of water

taken from spring and surface sources is shown in table 2.   The striking facts indicated in this table are first, the large number of negative results in the case of the spring samples and second, the large number of positive results in the case of samples taken from miscellaneous surface sources.

THE OCCURRENCE OF LEPTOSPIRAE IN WATER TAKEN AT VARIOUS STEPS IN THE FILTRATION PROCESS

It is also of interest to examine more closely the results obtained on cultivation of samples taken at various points in the water filtration plants of Baltimore and Washington and from taps in these cities.   These plants are essentially the same, in that they rely on rapid sand filtration.   The samples from these plants were taken from the raw water inlet, from the settling basins after the addition of alum, from the settling basins after the addition of alum and chlorine, from the collecting conduit after filtration subsequent to settling and the addition of lime, from the storage reservoir after filtration and subsequent chlorination and from the storage reservoir at the outlet to the city mains.   Other samples were taken from taps in the city some distance from the filtration plants.

Table 3 shows the source of the samples cultured, the step in the process of purification at which the samples were taken and the number of samples positive for leptospirae when these samples were incubated at body or room temperature.   The total number of samples from each source is not large, but we believe the results indicate the probable fate of leptospirae coming into the filtration plant in the raw water.   In the first place 50 per cent of the raw water samples yielded leptospirae, and 75 per cent of the samples treated with lime and alum alone were positive.   At the same time the single sample cultured subsequent to the alum treatment and sand filtration was positive while the filter bed washings were also positive, as would be anticipated.   However, it should be pointed out as highly significant that none of the samples examined after the water was chlorinated were positive, no matter whether the chlorine was added before or after filtration.   In a word, our results show that the addition of lime and alum, in the

proportions used in these filtration plants, is not lethal for leptospirae, but that the addition of chlorine as practiced is sufficient to free the water from these organisms.

Of greatest interest, however, are the results obtained from those samples of water taken from the tap in the laboratory or from similar sources in the city. Reference to table 3 will show that of these 13 samples 9 were positive. In other words, even though the water was freed of leptospirae during the filtration

TABLE 3

Indicating the occurrence of leptospirae in water samples taken at various points in two municipal filtration plants

SOURCE	TREATMENT	TOTAL NUMBER OF SAMPLES	NUMBER OF SAMPLES POSITIVE	NUMBER OF SAMPLES NEGATIVE
Raw	None	6	4	2
Settling basin	Treated with alum and settled	4	3	1
Settling basin	Treated with alum and chlorine	3	0	3
Filtered water	After rapid sand filtration	3	1	2
Filtered water	After rapid sand filtration, pre-chlorinated before filtration	4	0	4
Treated water at plant	After pre-chlorination, filtered and chlorinated again	7	0	7
Tap water	Water allowed to run for 5 min before sampling	13	9	4

process these organisms appear again in the water as it comes from the faucet. The explanation of this observation seems to us to lie in the probable growth and multiplication of these organisms in the mains.[1]

EVIDENCE OF THE EXISTENCE OF STRAINS

In the course of the dark-field examination of our cultures of leptospirae observations were made from time to time of variations in the morphology of the organisms that might have been

[1] Since this was written it has been suggested that the leptospirae might be growing in the secondary storage reservoirs but we have no evidence on this point.

readily interpreted as evidence that we were looking at different strains of these forms. However, experience with such variations occurring in many bacterial species under different conditions of cultivation makes us somewhat skeptical of reliance on this factor alone as evidence of difference in species. Consequently we believe that the results of our efforts to cultivate these organisms at room temperature and body temperature offer much more convincing evidence that we were dealing with different species.

With one exception all the samples that yielded leptospirae at body temperature were likewise positive at room temperature. Nor was the difference in growth in those samples cultured and found positive at both temperatures sufficiently pronounced to confirm in any sense the conclusion reached by Dimitroff that 25° to 28°C. is the optimum for these organisms.

On the contrary, it cannot be said that all the samples positive at room temperature were also positive at body temperature. It is from these results that we deduce the fact that we were dealing with different species. For example, in the case of sample 56, table 4, plates were made as has been indicated, two being incubated at each temperature. Examination of these plates after the usual period of incubation revealed leptospirae only in those incubated at room temperature. This observation being somewhat unusual we went back to the original sample and plated it out again, only to find that leptospirae appeared in the room temperature cultures as before. This procedure was repeated three times with the same results.   •

Similar observations were made on one of the samples of Washington tap water. The original plating yielded leptospirae only at room temperature, and in seven replatings from the original sample the same results were obtained. Furthermore, subcultures from these plates grew only at room temperature.

To us these results are convincing evidence that some strains differ in their temperature requirements for growth, and we infer that they represent different species. Such observations as these may throw some light on Walker's unsuccessful animal inoculation experiments in Washington. His cultures were grown at room temperature also and were inoculated into guinea pigs

without result. In the light of our cultivation experiments it would not seem strange that organisms which grow only at room temperature should fail to produce any reaction in warm blooded animals.

LONGEVITY OF LEPTOSPIRAE IN STORED SAMPLES OF WATER

In conclusion we wish to report some observations on the length of life of leptospirae in samples of water stored at room temperature in the laboratory.

The samples were not specially selected; the water was clear, with only a trace of such foreign substances as might get into the bottles when the samples were collected; the bottles were of clear glass with cotton stoppers and were kept on a shelf at room temperature exposed to the light. They were never in direct sunlight.

Table 4 shows the sources from which the samples came, the sample number, the date of collection, the result of the original plating in terms of the presence or absence of leptospirae at room or body temperature, the number of replatings made from the sample and the results of cultivation attempts after varying periods of storage. It will be seen that from 1 to 7 replatings were made. In numerous instances the number of replatings possible was conditioned by the amount of water in the original sample. It was also unprofitable, of course, to continue replating experiments in the case of any individual sample when the first replating was found negative.

In considering the results of replating after storage it should be emphasized that duplicate plates were incubated at both room and body temperature. This point is stressed because it is thought the results obtained may have a bearing on the question of the existence of species.

Reference to table 4 shows that of the samples positive on the original plating at 22°C. no. 79 was negative on replating after 14 days. This is the shortest period of storage which failed to yield leptospirae on replating at this temperature. On the contrary, the sample of Washington tap water originally positive at 22°C. still contained viable leptospirae when replated at this

TABLE 4

Showing the longevity of leptospirae in stored samples of water

SOURCE	SAMPLE NUMBER	DATE OF COLLECTION	RESULT OF ORIGINAL PLATING	NUMBER OF REPLATINGS	RESULTS AFTER STORAGE
Brook running through meadow receiving drainage from duck pond and cow pasture. Clear water	15	November 25, 1928	Positive at 22°C.	1	Negative after 42 days
	37	January 20, 1929	Positive at 22°C.	2	Positive at 22°C. after 30 days
Running stream. Drainage through tilled land and woodland. Clear water	16	November 25, 1928	Positive at 22°C.	1	Negative after 42 days
	39	January 20, 1929	Positive at 22°C.	1	Negative after 30 days
	56	February 18, 1929	Positive at 22°C.	3	Positive at 22°C. after 65 days
	83	April 8, 1929	Positive at 22°C.	1	Positive at 22°C. after 16 days
Gunpowder River	7	November 11, 1928	Positive at 22° and 27°C.	1	Negative after 46 days
	18	November 25, 1928	Positive at 22°C.	1	Negative after 42 days
	57	February 17, 1929	Positive at 22° and 37°C.	1	Positive at 22°C. and 37°C. after 21 days
	68	March 10, 1929	Positive at 22° and 37°C.	1	Positive at 22°C. and 37°C. after 21 days
Stream draining pasture	9	November 11, 1928	Positive at 22° and 37°C.	1	Negative after 45 days
	20	November 25, 1928	Positive at 22°C.	1	Negative after 42 days
	42	January 20, 1929	Positive at 22°C.	3	Positive at 22°C. after 50 days

Stream draining pasture	59	February 17, 1929	Positive at 22°C.	1	Negative after 29 days
	87	April 8, 1929	Positive at 22° and 37°C.	1	Positive at 22°C. and 37°C. after 16 days
Chinquepin Run. Decaying leaves and organic material	10	November 22, 1928	Positive at 22° and 37°C.	3	Positive at 22°C. after 62 days
	60	March 5, 1929	Positive at 22°C.	1	Negative after 21 days
Chinquepin Run. Drains area of woodland. ¼ mile from land that is pastured	2	October 5, 1928	Positive at 22°C	2	Positive at 22°C. after 34 days
	11	November 22, 1928	Positive at 22° and 37°C.	1	Negative after 28 days
	33	January 14, 1929	Positive at 22°C.	1	Negative after 28 days
Stream draining entirely wooded area	3	October 5, 1928	Positive at 22°C.	1	Positive at 22°C. after 34 days
	12	November 22, 1928	Positive at 22° and 37°C.	1	Negative after 45 days
	34	January 14, 1929	Positive at 22°C.	1	Negative after 29 days
	62	March 5, 1929	Positive at 22°C.	1	Negative after 27 days
	79	April 1, 1929	Positive at 22°C.	1	Negative after 14 days
Stream flowing through meadow. Certain amount of pollution from house probable	4	October 5, 1928	Positive at 22°C.	1	Positive at 22°C. after 34 days
	13	November 22, 1929	Positive at 22° and 37°C.	2	Positive at 22°C. after 61 days. Positive at 37°C. after 45 days
	92	April 15, 1929	Positive at 22°C.	1	Negative after 17 days

TABLE 4—*Concluded*

SOURCE	SAMPLE NUMBER	DATE OF COLLECTION	RESULT OF ORIGINAL PLATING	NUMBER OF REPLATINGS	RESULTS AFTER STORAGE
Stream draining cow pasture. Filled with grass, leaves, etc.	14	November 22, 1928	Positive at 22°C.	1	Negative after 45 days
	36	January 14, 1929	Positive at 22°C.	1	Negative after 42 days
	53	February 11, 1929	Positive at 22°C.	1	Positive at 22°C. after 23 days
	64	March 5, 1929	Positive at 22° and 37°C.	1	Positive at 22°C. after 27 days. Positive at 37°C. after 27 days
	81	April 1, 1929	Positive at 22°C.	1	Positive at 22°C. after 15 days
Running water in Clifton Park heavily polluted with kitchen wastes	27	December 16, 1928	Positive at 22° and 37°C.	3	Positive at 22°C. after 49 days. Positive at 37°C. after 20 days
Stagnant water at dump in Clifton Park	28	December 16, 1928	Positive at 22° and 37°C.	3	Positive at 22°C. after 65 days. Negative at 37°C. after 20 days
Running water in dump in Clifton Park. Filled with rusty cans, etc.	29	December 16, 1928	Positive at 22° and 37°C.	4	Positive at 22°C. after 97 days. Negative at 37°C. after 57 days
Standing water at dump on Echodale Avenue. Numerous rats in evidence	30	December 16, 1928	Positive at 22° and 37°C.	1	Negative after 20 days

Source	No.	Date	Result	No.	Result after days
Running water at dump on Echodale Avenue. Numerous rats in evidence	31	December 10, 1928	Positive at 22° and 37°C.	3	Positive at 22°C. after 49 days. Positive at 37°C. after 37 days
Chevy Chase tap water	71	November 17, 1928	Positive at 22° and 37°C.	1	Negative after 50 days
Montebello filtration plant. Raw water	25	March 22, 1929	Positive at 22°C.	1	Negative after 17 days
Montebello filtration plant. Settling tank	22	December 6, 1928	Positive at 22°C.	1	Negative after 30 days
Montebello filtration plant. Wash water from filter bed	24	December 6, 1928	Positive at 22° and 37°C.	4	Positive at 22°C. after 59 days. Positive at 37°C. after 29 days
Montebello filtration plant. Water after filtration		December 6, 1928	Positive at 22°C.	1	Negative after 30 days
Washington tap water		November 17, 1928	Positive at 22°C.	7	Positive at 22°C. after 109 days

temperature after 109 days storage. How much longer these organisms might have survived is unknown as the last replating used up the sample. At any rate this represents the longest period of survival in our experience.

Of the samples positive at 37°C. on the original plating, no. 28 was negative when replated at this temperature 20 days later. This represents the shortest period of storage which failed to yield leptospirae on replating at 37°C. In contrast to this, sample 13 was still positive when replated after 45 days storage and represents the longest period of viability for organisms growing at this temperature in our investigation.

In considering the negative results obtained on replating samples 79 and 28 after 14 and 20 days respectively, it is recognized that had replatings been made after shorter periods of storage we might have found the organisms had died out earlier. This is likewise true of other negative results recorded after varying periods of storage.

In the case of samples positive on the original plating at both temperatures, it is interesting to note that on replating they failed to show growth of leptospirae at 37°C. first in every case. Thus in sample 27, leptospirae viable at 22°C. were present after 65 days storage, but no growth at 37°C. was obtained after 20 days storage. In the case of sample 29 no growth on replating at 37°C. was obtained after 57 days storage, although organisms viable at 22°C. were still present after 97 days. Likewise in sample 31 the organisms growing out at 37°C. died out before the thirty-seventh day of storage, while those growing at 22°C. were still demonstrable after 49 days. Finally in sample 22 leptospirae growing at 37°C. were absent following 29 days storage of the sample, but those growing at 22°C. were still present after 59 days.

These differences in longevity of leptospirae cultivable at the two temperatures considered may be interpreted in two ways. First, they may indicate that there were really different species in our samples, or second, that cultivation at 22°C. is more nearly the optimum for growth of these organisms. We believe the first explanation is the correct one. This seems to be a logical

conclusion in view of the fact that in certain samples there were leptospirae cultivable only at 22°C. and that in others they always survived longer at this temperature than at 37°C.

SUMMARY

In this investigation water samples from 47 sources were examined for leptospirae growing at room or body temperature. A total of 131 samples were cultured. Seventy-three yielded leptospirae in our cultures; of these 40 were positive at 37°C. and 72 at room temperature.

In the case of samples taken from municipal filtration plants positive cultures were obtained from the water prior to chlorination only. They were found, however, in tap water after passage through the city mains.

Of the samples taken from springs none were positive.

As the result of our cultivation experiments, evidence of the existence of species in nature was obtained. This conclusion is based on the fact that leptospirae appeared in the cultures maintained at room temperature only and on the greater longevity of the organisms on recultivation from stored samples at this temperature.

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A CONTRIBUTION TO THE CLASSIFICATION OF MICROÖRGANISMS[1]

ERNST PRIBRAM

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I. INTRODUCTION; PLANTS, ANIMALS AND MICROÖRGANISMS

One of the most fascinating chapters in the study of unicellular organisms is that which deals with their relationship to the general realm of plants and animals. Their relatively simple morphology and biology offer us a wonderful opportunity for a consideration of phylogenetics. Without great effort one can trace the first stages of the development of the morphological and biological characteristics of unicellular organisms. They follow the same rules we find operative in the development of organisms with a higher differentiation of cells. The main difference, however, lies in the fact that the undifferentiated cell body is more exposed and more susceptible to the influence of the external world and the medium in which it develops.

There are four classes of unicellular microörganisms. Two of them, the Algae and the Fungi, have the characteristics of plants, the third, the Protozoa have the characteristics of animals. Between the Algae, the Fungi and the Protozoa the Schizomycetes stand as a continuous series of connecting links. Some of them have many characteristics of plants, others possess a few animal characteristics and are largely animal parasites. Many of the microörganisms have some characteristics of plants and some characteristics of animals; they are transitional forms. Some of these characteristics are unstable. They may be present or may not be present in the same species and even in daughter colonies which are derived from the same mother colony. The characteristics of plants which we find in the Fungi are different from those

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which we find in the Algae. The unicellular Fungi or Hypho-
mycetes have a protoplasmic body which contains but a small
amount of water. The cells form threads or hyphae, i.e., they
grow in one direction. They are isolated from the surrounding
medium by a resistant membrane which contains chitin. They
form resistant spores, they proliferate by budding and their
cells are branched. The metabolism of these Hyphomycetes is
based on the utilization of carbohydrates. The unicellular Al-
gae may be ovoid or they may form threads with sheaths. They
contain a water insoluble inorganic compound or element such as
sulfur or iron or red or brown or green pigments. These pigments
can be used in the absorption of certain rays of the light. The
green Algae have for example the same metabolism as green
plants have. They split the carbon dioxide of the air into carbon
and oxygen. They grow much better in the presence of light.

A typical Protozoon, an Amoeba for instance, has exactly
opposite characteristics. The soft protoplasmic body contain-
ing a large amount of water is directly exposed to the surrounding
fluid medium. It is only protected by the surface tension of its
protoplasmic body. This body has a spherical shape, if it is
resting, that is, if no external irritating influences change this
shape. A decrease of the tension of one part of the surface causes
a change in the equilibrium of the forces, which sustain this
surface tension. The parts with decreased tension are protruded,
they form pseudopodia and the body of the primitive animal
follows the pseudopodium in the direction of lowest resistance.
This exemplifies the primitive form of motility, one of the most
striking characteristics of animals. The food of animals is ac-
quired by active motility. This food consists of organized
material of plant and animal protoplasm, broken down by a
hydrolytic process, ordinarily called digestion. Protozoa do not
need light. They prefer the dark. They proliferate by cell
division.

The class of Schizomycetes has an intermediate position be-
tween the animal-like and the plant-like unicellular organisms.
Some of them have typical qualities of animals, others are en-
dowed with plant characteristics. Motility, a soft, Gram-nega-

tive body, a spherical colony shape, a proliferation by simple cell division, growth at 37° and the avoidance of the light are all animal characteristics. Spore formation, formation of water-insoluble pigments, production of substances, which have a high chemical resistance such as Gram-positive substances, acid-fast substances, granules, spores and cellulose formation are plant characteristics. Further qualities of plants are budding or ramification of the cells, thread formation, formation of ramified colonies and growth under anaerobic conditions. Among these main types there are again forms which have some qualities of the animal and others of the plant kingdom. If we trace the differences between the higher plants and the higher animals we understand, that these differences are based upon their different physico-chemical structure. The contents of plants consist of crystalloid solutions enclosed in a very resistant tissue. The protoplasm of plant cells contains a very small amount of water. It is protected from the external world by a resistant tissue, which is the cellulose. The main characteristics of aqueous crystalloid solutions are: A relatively low surface tension, a relatively low cohesion, i.e., a low mutual attraction of the molecules and therefore a tendency to expand and to increase the surface as much as possible. Since the fluid is enclosed in a dense tissue the expansion will follow one direction. The tendency to extend in this direction is increased by the adherence of the fluid to the wall of the tissue. All these conditions coöperate to produce a high capillary or hydrostatic pressure which is still more increased by the osmotic pressure of the crystalloids. The growth of plants follows this law of pressure which leads to an expansion in one direction. By this procedure they form roots, stems, branches, twigs and leaves. Plants need this large surface for their metabolism. They take their food from the soil and from the air. They have no active motility such as animals have. They are compelled to obtain their food by diffusion. The water ascends by capillary attraction. The carbon dioxide of the air is split by light in the presence of chlorophyll. Chlorophyll is a water insoluble pigment which utilizes the long waves of the light and reflects the short waves. Fermentation of carbohydrates, such

as dextrin is one of the prominent characteristics of plant metabolism. The metabolism of the seed differs essentially from the metabolism of the plant. Its primary growth is anerobic, air and light are excluded and a large quantity of fat is stored in a very resistant tegument. A fat splitting ferment enables the seed to utilize the fat. The plant seed buds and grows in one direction, thus starting its life as a plant.

The structure and development as well as the morphology and metabolism of animals is exactly the opposite to those of plants. This is due to the prevalence of the colloid character of the animal cell. The animal cell contains a protoplasm which is saturated with a limited quantity of water. Colloids have a high surface tension. They also possess a high cohesion of their molecules. They attempt to decrease their surface as much as possible. There is a great amount of energy confined within the smallest available space. The animal has its energy in its own body, the plant receives its energy from the long waves of light. Animals ferment organic food, i.e., the protoplasm and the other constituents of plants or animals. This is an exothermic process. It delivers energy. This free energy can be used for the production and the maintenance of the body temperature. It can also be transposed into other energies such as motility and electricity in the muscle and nerve tissue. Motility is the dominating feature of animal life. The intake of food, the action of the stomach and intestines upon the food, the intake of air, the blood circulation and the fertilization are caused by the active contraction of protoplasm. We have learned the conditions attending this contraction by the study of the simple protoplasmic body of the amoeba. We have learned there also that the protoplasmic cell of the animal is soft, that it is much more influenced by the external world than the plant cell. The reproduction by cell division causes a different shape than the reproduction by budding. If the cells do not separate after division they mutually influence their morphology. The tendency to retain the spherical shape of the surface, which is an important factor in the maintenance of surface tension, and which tends to be their main protection brings about a mere mutual connection. This process leads to

the formation of cavities with a large inner surface (Blastula, Gastrula). The process of invagination and infolding increases this inner surface. We can observe this procedure in the development of all parts of the animal body. The spherical shape is characteristic for the brain as well as for all the parenchymatous organs. Even the cardiac tube and the intestinal canal adapt their surfaces to the cavities in which they are suspended. The inner surface of all organs is enlarged as much as possible. This we find to be true in the tubuli and the glomeruli of the kidney, the glands and crypts of the intestines, the gyri of the brain, etc. The influence which the plastic protoplasmic cells exert on one another causes their high differentiation. Different stimuli irritate different cells and different parts of the cells and change their morphology and their metabolism. This is only true for the phylogenetic development of organs and tissues. The ontogenetic process is shorter and goes on without any external stimuli. We see for instance the development of the embryonic eye with a concomitant minute differentiation of its cells without the influence of light (Hertwig (1898)). The metabolism of the animal organism is maintained by the blood circulation. Here again we find an important chemico-physical difference between the blood vessels and their contents and the plant capillaries with their aqueous fluid. The blood plasm is a peculiar fluid with a large amount of proteins. It has opposite qualities to those of plant juice. The cohesion of the blood plasm is high; there is no adhesion to the wall of the vessels. The plant juice has a low cohesion and a high adhesion. It ascends slowly in the capillaries by its own pressure. The blood plasm passes the blood vessels and capillaries under a high speed and high pressure, pressed forward by the active contraction of the heart muscle.

The green chlorophyll is a water-insoluble substance, the red hemoglobin is water-soluble. The chlorophyll is a light filter for the long waves of the spectrum, the hemoglobin is a light filter for the short waves, which it passes, while the long waves are reflected. The animal cell avoids the light. We nevertheless know, that ultra violet light passes through the body and it can be accumulated in some parts of the body (Steenbock). The

red hemoglobin changes its color into the brown and green fluorescent bile. This substance, an intense sensibilizer for the light, is enabled to destroy toxins in a very short time in the presence of the short waves of light (Hausmann and Pribram 1909). The waste products of plants and animals are destroyed by microorganisms and the waste products of animals can be utilized by plants.

Summary

To recapitulate, the opposite character of plants and animals lies in this fundamental fact, that they have a different chemicophysical structure. Prevalence of crystalloids in plants, prevalence of colloids in animals. They further vary by their different separation from the external world, which is evidenced by cellulose membranes in plants, by surface tension in animals. They differ by the different development of their original cell which is followed by the corresponding development of tissues; growth in one direction, budding and branching in plants, equal growth in all direction in animals, resulting in a spherical shape of all animal organs. Budding in plants and fission in animals are the characteristic forms of proliferation. Splitting of carbon dioxide, utilization of the oxygen of the air, consumption of energy in plants, production of energy in animals with transposition into warmth, motility and other energies are fundamental differences in their metabolism. The long waves of the light are utilized by the green plants, the short ultra-violet rays by animals, and there are further striking contrasts in their metabolism. We shall see the same differences or at least many of them between the unicellular plant-like and animal-like microörganisms. We shall see that there are still many undifferentiated forms among them which have characteristics of both types. We are thus able to follow through the phylogenetic development of all these qualities in a complete series of organisms.

II. CLASSIFICATION OF THE CLASS SCHIZOMYCETES BASED UPON THEIR BIOLOGICAL RELATIONSHIP TO OTHER PLANTS AND ANIMALS

An exact international classification and nomenclature of bacteria is an urgent necessity, if we wish to continue our bacterio-

logical research work on a scientific basis. Linnée (1774) placed all microscopic organisms in a group and called them "chaos infusorium." Today, hundred and fifty years later we are in the position to classify this "chaos" applying Linnée's own fundamental principles of botanical nomenclature and classification. The rapid development of this science during the last 50 years has produced an immense literature on this subject. The detailed and very thorough classification and nomenclature of the Society of American Bacteriologists which is built on a real scientific basis ought to be developed into an international classification. Two standard works, Buchanan's General Systematic Bacteriology (1925) and Bergey's Manual of Determinative Bacteriology (1923) give us a clear survey of the literature. Studying these books we arrive at the conclusion, that Buchanan's clear outlines for the rules of an international botanical nomenclature do not need any further supplement. It is now necessary to develop a classification on the basis of such strict rules which should be adhered to in all scientific publications. It is imperative that a classification be both exact and elastic. In pointing out the common features of coördinated groups we have to characterize those things by which they are related. By labeling their main differences on the basis of common principles we can attain a high degree of definite terminology. Elasticity of classification can be secured by following the rule to use only one attribute as a characteristic of one group and to make subdivisions for each new characteristic. In following this rule we shall easily find a place for any of the microörganisms.

It is of course more or less arbitrary which characteristics we consider as the fundamental principles of our classification. We have selected those characteristics, which are closely related to the metabolism of the microörganisms. The metabolism of higher organisms depends largely on the morphological differentiation of their tissue cells. The metabolism of unicellular microörganisms depends on their accommodation to the medium on which or, in which, they live. The morphology of their colonies is a product of the surrounding medium to which they are adapted. The characteristics of one group (class, order, family, tribe, genus)

very often seem to take on to themselves similar or identical characteristics of other groups. We consider such forms "connective links" and try to give them an intermediate position among the groups, which agree with them in one or more features. The following classification and nomenclature is based upon the nomenclature of the Committee of the Society of American Bacteriologists. Numerous groups have been transferred from one position to another, and a few new names have been used which might be considered as tentative for the time being. Since I have followed Buchanan's Systematic Bacteriology and Bergey's Manual as guides for the main characteristics it will be necessary to give reasons only for divergences from these standard works.

There are four subclasses of the class Schizomycetes in our classification (See tabulation): *Protozoobacteria, Eubacteria, Mycobacteria* and *Algobacteria*. The Protozoa are unicellular organisms with a distinct nucleus and protoplasm. The nucleus of the Protozoobacteria is dissolved, forming the so called "Chromidia" or it at least expands and fills out the body of the organism. There is only one order, the *Spirochaetales*. They have many characteristics of the class Protozoa, but one of their families has also characteristics of the class Algae. The order Spirochaetales therefore might be considered as the connective link not only between the classes Protozoa and Schizomycetes but also between the classes Protozoa and Algae. The order Spirochaetales has two families. *The Spirochaetaceae* with an axis filament are related to the genus Trypanosoma, sub-class Mastigophora of the Protozoa (Doflein 1909). The second family of the order Spirochaetales, *the Cristispiraceae*, although closely related to the first order has some characteristics of the genus Oscillaria of the class Algae (G. Schmid, 1922). We could call them Algobacteria as well as Protozoobacteria. We see here a striking transition form between two classes of microörganisms.[2] We subdivide the family Spirochaetaceae according to the ab-

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[2] The author has described another striking form in a previous paper (1924, 1925): the *Torula nigra* which is a transition form between yeasts and fungi. (Saccharomycetes and Hyphomycetes).

TABULATION

Class: *Schizomycetes.* Unicellular microörganisms, which divide by fission
Subclass 1: *Protozoobacteria.* Connective link with Protozoa
Order
Spirochaetales
Flexuous spirals without flagella. Motile by oscillation

Family 1

Spirochaetaceae
Axis filament present.
Genus 1
Spirochaeta
Without septa, without membrane, without crista, without terminal filament.
Genus 2
Treponema
With terminal filament, without septa, membrane and crista.
Genus 3
Spironema
With membrane and terminal filament; without septa and crista.

Family 2

Cristispiraceae
Axis filament absent. Connective link with Oscillaria (Alga).
Genus 1
Saprospira
Septa and membrane present, crista absent.
Genus 2
Cristispira
Septa, membrane and crista present.
Genus 3
Leptospira
Position doubtful. Without septa, membrane and crista. (Axis filament probably absent.)

Subclass 2: *Eubacteria.* "*True Bacteria.*" Primitive rigid cells. Multiply only by transverse fission
Class: *Schizomycetes*
Order 1
Protobacteriales
Autotrophic

Family 1

Nitrobacteriaceae
Oxidizing Hydrogen, Carbon, Nitrogen or their inorganic compounds. Closely related to the **Pseudomonas.**

Family 2

Thiobacillaceae
Utilizing Sulphur or its inorganic compounds.

Tribe:

Tribe 1
Hydrogenomonadeae
Utilizing Hydrogen and Carbon.
Genus 1
Hydrogenomonas
   Utilizing Hydrogen.
Genus 2
Methanomonas
   Utilizing Carbon and Hydrogen.
Genus 3
Carboxydomonas
   Utilizing Carbon.

Tribe 2
Nitrobactereae
Utilizing Nitrogen.
Genus 1
Nitrosomonas
   Oxidizing Ammonia.
Genus 2
Nitrobacter
   Oxidizing Nitrites.

Thiobacilleae
Acting on Sulphur.

Genus:
   Thiobacillus

Order 2
Metabacteriales
Heterotrophic

Family 1

Pseudomonadaceae
Metabolism adapted to soil and water. Gram negative. Motile by means of polar flagella. Many forms curved.

Family 2

Bacteriaceae
Metabolism adapted to the metabolic products of plants and animals. Straight Gram negative rods. Flagella peritrichous.

Family 3

Micrococcaceae
Metabolism adapted to certain products of the animal metabolism (e.g. milk); few water forms.
                Spheric cells

Tribe 1 Spirilleae	Tribe 2 Vibrioneae	Tribe 3 Pseudomonadeae	Tribe 1 Aerobactereae	Tribe 2 Pasteurelleae	Tribe 1 Streptococceae	Tribe 2 Micrococceae
Cells spiral.	Curved rods.	Straight rods with a tendency to become curved.	Ferment carbohydrates.	Do not ferment carboh.: or have very slight ferments for carboh.	Cell division in one direction.	Cell division in different directions: Gram positive.
Genus: Spirillum	Genus: Vibrio.	Genus 1 *Pseudomonas*	Genus 1 *Aerobacter* Producing acetyl-methyl-carbinol.	Genus 1 *Alcaligenes* Litmus milk unchanged or alkaline or slimy.	Genus 1 *Neisseria* Gram negative diplococci. Strict parasites.	Genus 1 *Micrococcus* Cell division in plates.
		Var. a Reducing Nitrates to Nitrogen or Ammonia.	a. motile: *Aerobacter.*	a. Motile: *Alcaligenes.*	Genus 2 *Streptococcus* Chain formation in one direction.	Genus 2 *Staphylococcus* Cell division in different planes in irregular masses or in chains.
		Var. b Reducing Nitrates to Nitrites.	b. non motile: *Aerogenes.*	b. Non motile: *Brucella.*	a. Gram positive.	Genus 3 *Sarcina* Rectangular fission in 3 planes.
		Var. c Nitrates not reduced.	Genus 2 *Escherichia* Ferment lactose, coagulate milk.	Genus 2 *Pasteurella* Showing bipolar staining. Form pellicle on broth. Connecting link with Pfeifferella. Non motile.	b. Gram negative.	Connective link with Algobacteria.
		Genus 2 *Azotobacter* Capable of fixing Nitrogen. Connective link with Polyangiaceae, also with Nitrobacteriaceae.	a. motile: *Escherichia.*	Genus 3 *Hemophilus* Strictly adapted to the ani-		
			b. non motile: *Encapsulatea.*			
			Genus 3 *Salmonella* No gas from lactose but from other carboh. Milk acid or unchanged turns later			

Genus 4
Eberthella
Acid but no gas from some carboh.
a. motile: *Eberthella.*
b. non motile: *Shigella.*
Genus 5
Proteus
Ferment sucrose, putrefy proteins. Pleomorphic. Motile.

Growing only in the presence of highly differentiated compounds. Connecting link with **Dialister.** Non motile.

Class: Schizomycetes

Subclass 3: Mycobacteria. Connective link with Eumycetes

Order 1

Bacteriomycetales

Unbranched straight and curved rods or filaments. Surface colonies on liquid media unbranched "mycodermata." No entire. Surface colonies on solid media filamentous, curled or arborescent, never spores formed. Connective link with Eubacteria

Family 1
Leptotrichaceae
Aerobic
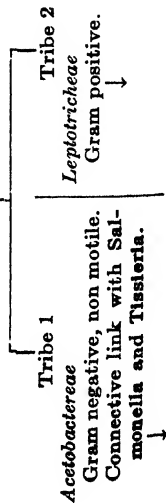
Family 2
Bacteroidaceae
Anaerobic

Tribe 1
Acetobactereae
Gram negative, non motile. Connective link with Salmonella and Tissieria.
→

Tribe 2
Leptotricheae
Gram positive.
→

Tribe 1
Dialistereae
Gram negative.
→

Tribe 2
Bacteroideae
Gram positive.
→

Genus:
Acetobacter
Form acetic acid from alcohol.

Genus 1
Kurthia
Motile by means of peritrichous flagella.
Genus 2
Lactobacillus ("Plocamobacterium")
Non motile. Straight and curved rods.
Connective link with **Corynebacterium.**
Genus 3
Leptotrichia
Non motile filaments.
Connective link with **Erysipelothrix.**

Genus 1*
Type species: *Dialisterea variegata.*
Motile.
Connective link with **Distasoa.**
Genus 2*
Type species: *Dialisterea variabilis.*
Non motile; saprophytic.
Genus 3
Dialister
Non motile: strict parasite.
Growth only in the presence of fresh tissue.
Connective link with **Hemophilus.**

Genus 1*
Type species: *Bacteroidea multiformis.*
Motile.
Genus 2
Bacteroides†
Non motile. Straight short rods with round ends.
Connective link with **Tissieria.**
Genus 3*
Type species: *Bacteroidea fusiformis.*
Non motile. Curved rods with pointed ends.

* Name vacant.
† Type species: Bacteroides fragilis.

Spore formers. Surface colonies on solid media arborescent, never entire. Aerobic forms form pellicles on the surface of liquid media. Unbranched rods. Connective link with Ascomycetes

Order 2
Bacillomycetales

Family 1
Bacillaceae
Aerobic

Family 2
Clostridiaceae
Anaerobic
Gram positive

Subfamily 1
Aerobacilloideae
Gram negative

Subfamily 2
Bacilloideae
Gram positive

Subfamily 1
Botulinoideae
Motile, peritrichous flagella

Subfamily 2
Clostridioideae
Non motile

Tribe 1 Aerobacilleae Motile, peritrichous flagella.
Subtribe 1 *Centrosporineae* Spores central or excentric.
Genus 1 *Centrosporus* Rods swollen at sporulation.
Genus 2 *Fusibacillus** Rods not swollen at sporulation.
Subtribe 2 *Aerobacillineae* Spores terminal or subterminal.
Genus: *Aerobacillus†* Rods swollen at sporulation.

Tribe 2 Pseudobacilleae Motile, polar flagella.
Genus: *Pseudobacillus‡* Spores terminal.

Tribe 1 Bacilleae Motile, peritrichous flagella.
Subtribe 1 *Bacillineae* Spores central or excentric.
Genus 1 *Bacillus* Rods swollen at sporulation.
Genus 2 *Megatherium* Rods not swollen at sporulation.
Subtribe 2 *Astasineae* Spores terminal or subterminal.
Genus 1 *Astasia* Rods swollen at sporulation.

Tribe 2 Anthraceae Non motile.
Genus: *Anthrax* Spores central. Rods not swollen at sporulation.

Tribe 1 Botulineae Spores central or excentric.
Genus 1 *Botulinus* Rods swollen at sporulation. Brain blackened.
Genus 2 *Chauvoea* Rods swollen at sporulation. Brain not blackened.
Genus 3 Type species: *Botulinea saccharolytica.* Rods not swollen at sporulation. Brain blackened.

Tribe 2 Putrificeae Spores terminal or subterminal.
Genus: *Putrificus* Rods swollen at sporulation. Brain blackened.

Tribe 1 Welchieae Spores central or excentric.
Genus: *Welchia* Rods not swollen at sporulation. Brain blackened.

Tribe 2 Clostridieae Spores terminal or subterminal.
Genus: *Clostridium.* Rods not swollen at sporulation. Brain blackened.

Genus 2
Flexus
Rods not
swollen
at spor-
ulation.

Genus 4
Type
species:
Botulinea
butyrica.
Rods not
swollen
at spor-
ulation.
Brain not
black-
ened.

* Type species: Fusibacillus fusiformis (Gottheil). Spores also terminal (Ford).
† Type species: Aerobacillus terminalis.
‡ Type species: Pseudobacillus macerans. Gram negative (Buchanan), *not* Gram positive (Bergey).

Order 3
Actinomycetales
Branched rods or branched filaments. Connective link with Hyphomycetes

Family 1
Mycobacteriaceae
Without exospores

Family 2
Actinomycetaceae
With exospores (Conidia)

Tribe 1
Actinobacilleae
Aerobic. Not acid-
fast.
Genus 1
Pfeifferella
Gram negative.
Rod shaped.
Connective link
with Pasteurella.

Tribe 2
Mycobactereae
Aerobic; acid-fast.
Genus 1
Phleobacterium
Saprophytic.
Genus 2
Mycobacterium
Parasitic.

Tribe 3
Tissierieae
Anaerobic. Not acid-
fast.
Genus 1
Distasoa*
Motile.
Gram negative.
Connective link
with Variegatus
(Dialistereae).

Tribe 1
Actinoidomycetaceae
Gram negative.
Genus:
Actinoidomyces‡

Tribe 2
Actinomycetaceae
Gram positive.
Genus:
Actinomyces

Genus 2
Actinobacillus
Gram negative
filaments.
Genus 3
Corynebacterium
Gram positive.
Rod shaped.
Connective link
with Lactobacillus.
Genus 4
Erysipelothrix
Gram positive
filaments.
Connective link
with Leptotrichia.

* Type species: Distasoa bullosa.
† Type species: Tissieria bifida.
‡ Type species: Actinoidomyces actinoides.

Genus 2
Tissieria†
Non motile.
Gram positive.
Connective link
with Bacteroides,
with Corynebac-
terium and with
Acetobacter.

Class: Schizomycetes

Subclass 4: Algobacteria. Connective link with Algae

Order 1
Desmobacteriales
Filaments surrounded by a firm sheath. No Iron or Sulfur deposits

Family
Sphaerotilaceae
Multiplication by gonidia

Genus Sphaerotilus
Motile and non motile gonidia
Connective link with Clonothrix

Order 2
Siderobacteriales

Sheathed or encapsulated cells containing deposits of Iron in their sheaths or capsules

Family 1
Chlamydotrichaceae
Filaments

Tribe 1
Chlamydotricheae
Unbranched.

Genus 1
Leptothrix
(= Chlamydothrix
Migula.) Filaments
not attached.

Genus 2
Crenothrix
Filaments attached.

Tribe 2
Clonotricheae
Branched.

Genus:
Clonothrix
Multiplies by non motile
gonidia.
Connective link with
Sphaerotilus.

Family 2
Siderocapsaceae
Short cells

Genus 1
Didymohelix
Curved cells excreting filaments, which contain Iron.

Genus 2
Siderocapsa
Short rods or cocci enclosed in a zoogloea, surrounded by
an area which contains Iron.

Genus 3
Sideromonas
Short rods or cocci forming chains. The chains are en-
closed in a dense capsule, which contains Iron.

Order 3
Thiobacteriales
Containing granules of free sulphur or bacteriopurpurin or both

Family 1
Rhodobacteriaceae
Cells containing bacteriopurpurin

Subfamily 1
Chromatoideae
Cells containing sulphur granules.

Tribe 1
Thiocapseae
Cells united into families. Cell division in 3 directions of space.

Genus 1
Thiocystis
Cells capable of swarming. Families enveloped in a gelatinous cyst.

Genus 2
Thiosphaera
Cells swarming. Loosely bound by gelatin.

Genus 3
Thiosphaerion
Cells swarming. United into solid spherical families.

Subfamily 2
Rhodobacteroideae
Cells without sulphur granules.

Tribe 1
Rhodobacteriaceae
Cells free.

Genus 1
Rhodobacterium
Elongate straight non motile cells.

Genus 2
Rhodobacillus
Elongate straight motile cells.

Genus 3
Rhodovibrio
Comma shaped cells with polar flagellum.

Genus 4
Rhodospirillum
Spiral cells with polar flagella.

Family 2
Beggiatoaceae
Non motile filaments containing sulphur granules, *no* bacterio-purpurin.

Genus 1
Thiothrix
Non motile, attached. Differentiation into base and tip.

Genus 2
Beggiatoa
Motile (oscillating) free filaments not attached not differentiated.

Genus 3
Thioploca
Motile (oscillating) filaments in bundles surrounded by a gelatinous sheath.

Family 3
Achromatiaceae
Motile cells, not filamentous containing sulphur granules, no bacterio-purpurin.

Genus 1
Achromatium
Spherical later ellipsoidal cells containing granules of calcium oxalate, perhaps sulphur.

Genus 2
Thiophysa
Spherical cells with sulphur granules in a central vacuole.

Genus 3
Thiospira
Spiral cells.

Genus 4
Hillhousia
Large long cells with peritrichous flagella.

Genus 4
Thiocapsa
Not swarming. Families loosely connected by gelatin.

Genus 5
Thiosarcina
Not swarming. Arranged in regular packets.

Genus 6
Lamprocystis
Swarming. Cell division in 3, then in 2 directions of space.

Tribe *2*
Thiopedieae
Cells united into families. Cell division in two planes.

Genus 1
Lampropedia
Cells united into tetrads.

Genus 2
Thioderma
Cells occurring in a membrane.

Tribe *3*
Amoebobactereae
Cells united into families. Cell division in one plane.

Genus 1
Amoebobacter
Cells connected by plasma threads.

Genus 5
Rhodosphaera
Spherical cells.

Tribe *2*
Rhodocysteae
Cells not free.

Genus 1
Rhodocystis
Rod shaped cells in a slimy cyst.

Genus 2
Rhodonostoc
Spherical cells in chains, chains surrounded by a capsule.
Connective link with Leuconostoc.

Genus 2
  Thiodyction
    Cells arranged in a net.
Genus 3
  Thiothece
    Cells loosely aggregated in gelatin.
Genus 4
  Thiophylococcus
    Cells appressed into a colony.
Tribe 4
  Chromatieae
    Cells free, swarming.
Genus 1
  Chromatium
    Cylindrical or elliptical cells with polar flagella.
Genus 2
  Rhabdomonas
    Rod shaped or spindle shaped motile cells.
Genus 3
  Thiospirillum
    Spiral motile cells.
Genus 4
  Rhodocapsa
    Non motile.
Genus 5
  Rhodothece
    Non motile, encapsulated.

Order 4
Myxobacteriales
Producing a gelatinous aggregation. Later becoming encysted

Family 1

Polyangiaceae
Rods shorter, when encysted, but not transformed into coccus like spores.
Genus 1
Chondromyces
Forming free cysts.
Genus 2
Polyangium
Forming cysts within a gelatinous mass.

Family 2

Myxococcaceae
Rods transformed into coccus like spores when encysted.
Genus:
Myxococcus

sence or presence of septa, membrane, crista and terminal filament into three genera and the family Cristispiraceae on the basis of the same fundamental principle into three genera; the position of the last genus is doubtful, because it is uncertain whether it possesses an axis filament or not. (Tabulation.)

The second subclass, *Eubacteria*, or "true Bacteria" are primitive rigid cells multiplying *only* by transverse fission. There is no other form of reproduction, no spore formation and no budding in this subclass. According to their metabolism they can be subdivided into two main orders, *Protobacteriales* and *Metabacteriales*. The Protobacteriales are autotrophic. They are capable of securing energy by direct oxidation of hydrogen, carbon, nitrogen, sulfur or of inorganic compounds of these elements. Their first family, the *Nitrobacteriaceae* oxidize hydrogen, carbon or nitrogen or their inorganic compounds. They are typical soil bacteria and closely related to the Pseudomonas. Their motile forms have polar flagella and especially the forms which act on nitrogen (tribe Nitrobactereae) are related to the genera, Pseudomonas and Azotobacter (of the tribe Pseudomonadeae). The main difference in their metabolism is this, that the Nitrobactereae oxidize nitrites (Nitrobacter) or ammonia (Nitrosomonas), while the Pseudomonadeae reduce nitrates (Pseudomonas) or fix nitrogen (Azotobacter). Another difference in their metabolism is this, that the Nitrobactereae do not grow in the presence of certain metabolic products of higher organisms, such as glucose. The second family, the *Thiobacilleae*, act on sulphur and its inorganic compounds. These autotrophic forms might have been the first organisms on our earth.

The second order of the subclass Eubacteria are the *Metabacteriales*. They are heterotrophic. They utilize metabolic products of other organisms, i.e. organic compounds of carbon, hydrogen, nitrogen, such as carbohydrates and proteins and their derivatives. They are subdivided into three families. The Pseudomonaceae are Gram negative rods, motile by means of polar flagella. A few forms are non-motile by loss of flagella. Many forms are curved. Their metabolism is adapted to soil and water. Their tribes are *Spirilleae*, spiral shaped, *Vibrioneae*,

curved rods, and *Pseudomonadeae,* straight rods. There is also a tendency to form curved rods in the tribe Pseudomonadeae. Pribram and Pulay (1915) found, that the same culture of the species *Pseudomonas fluorescens* grew as straight rods at 37° and as curved rods (vibrios) at room temperature. The tribe Pseudomonadeae includes all the Gram-negative non-spore formers which possess polar flagella, such as the genus Phytomonas and a part of the genus Flavobacterium of Bergey's Manual. It also includes some forms of the genus Alcaligenes of Bergey's classification. (Pribram and Monias, 1927, 1928). The genus Pseudomonas has three subdivisions: forms, which reduce nitrates to nitrogen or ammonia, forms which reduce nitrates to nitrites and those forms which do not reduce nitrates. Many of these forms produce a water-soluble pigment (*Pseudomonas fluorescens*) or water soluble pigments (*Pseudomonas pyocyanea*). The presence of a water soluble pigment however is not an essential characteristic of this genus. The second genus of the tribe Pseudomonadeae is Azotobacter, which is capable of fixing nitrogen. It is, as we explained, the connective link with the tribe Nitrobactereae of the order Protobacteriales. One of the species of the genus Azotobacter, the *Azotobacter rhizobius,* is also closely related to the genus Polyangium of the subclass Algobacteria (see also Pinay: Lehmann and Neumann, 1927). They are therefore transition forms between the Eubacteria and the Algobacteria.

The second family of the order Metabacteriales are the *Bacteriaceae.* They are Gram-negative straight rods. Their metabolism is adapted to the metabolic products of plants and animals. The motile forms possess peritrichous flagella. Some of the non-motile forms have the tendency to form capsules: Aerogenes, Encapsulatea, (*Pasteurella pestis*). The first tribe of the family Bacteriaceae, the *Aerobactereae,* is characterized by strong ferments for carbohydrates. The first genus of the tribe Aerobactereae, *Aerobacter,* produces acetylmethylcarbinol. There are two subgenera, the motile form, Aerobacter and the non-motile form, Aerogenes. The second genus, *Escherichia,* ferments lactose with gas formation and coagulates milk. It does not pro-

duce acetylmethylcarbinol. There exists also a non-motile form of this genus, the subgenus *Encapsulatea*. The third genus of the tribe Aerobactereae, *Salmonella*, forms acid and gas from some carbohydrates, but no gas from lactose. There is no production of acetylmethylcarbinol in this tribe. The milk culture might be unchanged or acid. It later turns usually alcaline. There may also be non-motile forms, but they seem to be rare. Many species of the tribes "Chromobactereae" and some of the "Achromobactereae" of Bergey's Manual belong to Aerobacter or to Salmonella: *Salmonella marcescens* or *prodigiosa*, *Salmonella agilis* etc. Other forms of the tribes Chromobactereae and Achromobactereae belong to the genus Proteus, a few of them belong to the genus Alcaligenes or they are Pseudomonadeae. (Compare the thorough studies on this subject by Breed and others, 1926, 1927, 1928). The fourth genus of the tribe Aerobactereae, *Eberthella* forms acid but no gas from some carbohydrates. The non-motile subgenus of this genus might be called *Shigella*. (Castellani.) The fifth genus, *Proteus*, has only motile forms. It ferments sucrose and acts on proteins.

The second tribe of the family Bacteriaceae is the tribe *Pasteurelleae*. Its first genus, *Alcaligenes*, does not act on litmus milk or turns it alcaline or slimy. There are motile forms and the non-motile subgenus *Brucella*. The second genus, *Pasteurella*, which has very weak ferments for carbohydrates, is characterized by a special differentiation of its cell body, a bipolar staining. The differentiation of the cell body by the formation of granules etc. is one of the characteristics of higher or more differentiated forms, as we shall learn later. It is characteristic for the Corynebacteria for instance. These as well as Pfeifferella, which is closely related to the genus Pasteurella are branched and are placed in our classification in the family Mycobacteriaceae of the subclass Mycobacteria. Pasteurella is therefore one of the connective links between the Eubacteria and the Mycobacteria. We shall learn, that there are some other connective links between these subclasses. The third genus *Hemophilus*, is strictly adapted to animal metabolism. It grows only in the presence of highly

differentiated compounds. It is the connective link with the "Dialister" (Bacterio-mycetales). It is non-motile.

The third family of the order Metabacteriales are the Micrococ-caceae, spherical cells.[3] Their first tribe, the *Streptococceae* multiply by division in only one direction. They have two genera: The *Neisseria*, Gram-negative diplococci, strict parasites, and the *Streptococcus* with Gram-positive and Gram-negative forms. These organisms are also adapted to the animal metabolic products, such as milk, blood, serum, pus. Other forms have strong ferments for carbohydrates. There are also transitional forms to the Algobacteria in this genus: the *Streptococcus leuconostoc*, which is related to the Rhodonostoc. The Gram-positive staining of the cell body of some of the subvarieties of the streptococci also indicates the transitional character of this family, which is more distinct in the next tribe, the *Micrococceae*. They are characterized by cell division in different directions, either in one plane (in plated or irregular masses), as is found in *Micrococcus*, or in chains in different planes or in irregular masses in different planes, as in *Staphylococcus*, or by rectangular fission, as in *Sarcina*. All these forms are Gram-positive and pigmented, both characteristics of the more highly differentiated forms. The Gram-positive character of the cells indicates a high resistance. There are other facts, which agree with this idea. Gram-negative forms are soluble in a concentrated solution of potassium thiocyanate, Gram-positive forms are insoluble in this solution (Porges, unpublished paper). The production of a cell body having a high resistance is one of the characteristics of plants. The closer the microörganisms approach to the fungi, the more Gram-positive forms we find, and the more resistant forms, such as granules, spores, acid fast bacteria, even real cellulose formation (*Acetobacter xylinus*). The production of a pigment which is water-insoluble and fixed in the cell body is one of the first attempts of the unicellular organ-

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[3] The name Coccaceae used in Bergey's Manual should be replaced by the name Micrococcaceae. Article 21 of the code of botanical nomenclature specifies, that families are to be designated by the name of one of their genera or ancient generic names. There is no bacterial genus Coccus (Buchanan, l.c. p. 271). We suggest for this reason the name Micrococcaceae.

ism to utilize certain rays of the spectrum. This phenomenon later leads up to the production of chlorophyll which is so important in the metabolism of plants. The Micrococci, Staphylococci and Sarcinae with their Gram-positive character, and especially the pigmented forms, are transition forms which connect the Eubacteria with the Algobacteria.

The third subclass of the class Schizomycetes, the *Mycobacteria*, includes all unicellular microörganisms possessing characteristics which we find in the class Eumycetes. The first of the three orders of the subclass Mycobacteria is still closely related to the Eubacteria. We suggest the name *Bacteriomycetales* for this order. They develop unbranched mycelia or "mycodermata" on the surface of liquid media. Their superficial colonies on solid media are either filamentous, curled or arborescent. They never are spherical as the colonies of the Eubacteria always are. They further have the tendency to form long unbranched rods or even filaments. This order and all the following orders of the Mycobacteria have, besides the Gram-negative forms, also Gram-positive genera and, besides aerobic, also anaerobic ones. We subdivide the order Bacteriomycetales into two families, the aerobic *Leptotrichaceae* and the anaerobic *Bacteroidaceae*. The first tribe of the family Leptotrichaceae, the *Acetobactereae* are Gram-negative non-motile rods. They are related to the genus Salmonella. This relationship is indicated by their Gram-negative character, and by their action on carbonaceous compounds, such as sugar and acetic acid. The differentiation can easily be made by the pellicle formation on the surface of liquid media, the tendency to form chains, the presence of club-shaped forms etc. It is very significant, that one of the species of the genus Acetobacter, the *Acetobacter xylinus* has a slimy envelope, which gives the cellulose reaction. The formation of resistant cellulose is one of the typical plant characteristics. The second tribe of the family Leptotrichaceae the tribe *Leptotricheae* contains the Gram-positive forms of the family. The first genus, *Kurthia*, is motile. It is related to the genus Proteus, which might be called the Gram-negative motile form of the family Leptotrichaceae. We prefer to place Proteus with the Eubacteria on account of its strong

fermentative power, its motility and its Gram-negative staining. The genus Proteus is a typical transition form, connecting the subclasses Eubacteria with the Mycobacteria. The second and third genera of the tribe Leptotricheae are non-motile. Genus 2, *Lactobacillus*, Gram-positive rods, corresponds to the Gram-negative Acetobacter. Lehmann rejects the name Lactobacillus and accepts the name Plocamobacterium, suggested by one of my pupils, E. Loewi (1920). Although we agree with Lehmann and Neumanns' arguments (1927) we hesitate to use this name, which is very characteristic, because we follow in this paper strictly the botanical codes of nomenclature and avoid therefore the temptation to replace the older generic names by new ones. However we recommend the name Plocamobacterium ("braid like colonies," plocamon, braid). The reasons for this name are given by Lehmann. (Bakteriologische Diagnostik, p. 506.) Some species of the genus Lactobacillus form granules in their bodies (*Lactobacillus bulgaricus*). This again shows the tendency of these forms to produce resistant corpuscles as the genera Corynebacterium and Pfeifferella do. These genera are placed to the family Mycobacteria on account of their branching. The third genus of the tribe Leptotricheae, the *Leptotrichia*, has non-motile filaments. It does not act on carbohydrates as the Lactobacillus does. It is related to the Algobacteria and might be considered as the transition form to the subclass Algobacteria. It is a facultatively aerobic form, but some forms grow better under anaerobic conditions.

The second family of the order Bacteriomycetales are the anaerobic *Bacteroidaceae*. This is the first strictly anaerobic family which we meet in our classification. The anaerobic growth is a typical characteristic of plants. The seed implanted in the soil is budding and growing under anaerobic conditions. The family Bacteroidaceae are not spore formers and are unbranched according to the characteristics of the order Bacteriomycetales. Their Gram-negative tribe, the *Dialistereae*, can be subdivided into motile and non-motile genera (see tabulation). The names of these genera are vacant. They are replaced by the type species. Dialister is the connective link with the Hemophilus

(Eubacteria). The Gram-positive tribe, the *Bacteroideae* have again motile and non-motile genera. The non-motile forms are either straight rods with round ends, like the type species *Bacteroides fragilis*, or they are curved rods with pointed ends, such as the type species *Bacteroidea fusiformis*. The type species of the motile genus is *Bacteroidea multiformis*. The use of the name of the tribe as a generic name might be allowed until a definite name for each of these genera is settled.

The second order of the subclass Mycobacteria, the *Bacillomycetales*, are spore formers. Spore formation means a new form of reproduction, namely that of budding. Spore formation and budding are characteristics of the Eumycetes. The Bacillomycetales are related to the spore forming Eumycetes, the Ascomycetes (compare Arthur Meyer, quoted by Lehmann). Their aerobic family are the *Bacillaceae*. The first tribe of this family may be called *"Aerobacilleae."* They are Gram-negative rods, motile by means of peritrichous flagella. The second tribe, *"Pseudobacilleae"* are Gram-negative rods motile by means of polar flagella. They correspond to the Pesudomonadeae of the subclass Eubacteria. The third tribe are the *"Bacilleae,"* Gram-positive rods, motile by means of peritrichous flagella. The fourth tribe are Gram-positive, non-motile rods, the *"Anthraceae."* The subtribes and the genera with the characteristics of their spore formation can be traced in the tabulation. They correspond to the classification of Bergey's Manual. The names are merely tentative. The anaerobic family of the order Bacillomycetales are the *Clostridiaceae*. The two subfamilies of the Clostridiaceae, subdivided according Bergey's Manual, are the motile *"Botulinoideae"* and the non-motile *"Clostridioideae."* The further subdivision of our tabulation follows exactly Bergey's classification. The generic names: *"Botulinus," "Putrificus," "Welchia," "Chauvoea"* are tentative names. They might be replaced by the type species, if there should be any objection against the formation of new generic names.

The third order of the subclass Mycobacteria are the *Actinomycetales*. This order includes all the branched forms of the subclass Mycobacteria. Branching means budding, the form of pro-

liferation, which is characteristic for the class Eumycetes. There are two families in the order Actinomycetales. The first family are the *Mycobacteriaceae* without spores. ' The second family are the *Actinomycetaceae* with exospores ("conidia"). This family is closely related to the Hyphomycetes. The not acid-fast aerobic tribe of this family, the *Actinobacilleae*, has four genera, namely the Gram-negative rod-shaped *Pfeifferella*, the Gram-negative filamentous *Actinobacillus*, the Gram-positive rod-shaped *Corynebacterium*, and the Gram-positive filamentous *Erysipelothrix*. The relationships of Pfeifferella with Pasteurella and of Corynebacterium with Lactobacillus have been mentioned. The anaerobic tribe of the family Mycobacteriaceae, might be called Tissiereae. There are two genera so far known of this tribe: the Gram-negative, motile *Distasoa* with the type species *Distasoa bullosa* and the Gram-positive non-motile *Tissieria* with the type species *Tissieria bifida*. Both forms are branched and we place them therefore in the order Actinomycetales, which contains all the branched forms of the sub-class Mycobacteria. We find them in Bergey's Manual treated with the other Bacteroideae. Lehmann emphasizes the relationship of *Bacterium bifidum* to *Corynebacterium diphtheriae* as well as its relationship to Acetobacter. The third tribe of the family Mycobacteriaceae are the acidfast *Mycobactereae*. Only Gram-positive aerobic forms of this tribe are known: the saprophytic genus *Phleobacterium* and the parasitic genus *Mycobacterium*. The second family, the Actinomycetaceae is characterized by the formation of conidia. It is the transition form to the *Hyphomycetes*. We distinguish two tribes: the Gram-negative *Actinoidomyceteae* and the Gram-positive *Actinomyceteae*.

The fourth subclass, the *Algobacteria* contains those forms which are related to the class Algae. We suggest for the first order of the subclass Algobacteria the name *Desmobacteriales*. The name Desmobacteria, used first by Cohn in 1872, has been adopted by Hansgirg (1888) for the families Cladotrichaceae, Crenotrichaceae and Leptotrichaceae. (cf. Buchanan, l.c.p. 288.) The Desmobacteriales are filamentous cells with a firm sheath. They do not deposit iron or sulphur. Only one family

of this genus the *Sphaerotilaceae* is known. It multiplies by means of gonidia. It has only one genus *Sphaerotilus* with motile and non motile gonidia. It is closely related to the genus Clonothrix of the order Siderobacteriales.

The second order of the subclass Algobacteria are the *Sidero-bacteriales*. The deposit of iron is an important biological step forward in the development of plant characteristics. The question, whether the inorganic iron compounds can be utilized for the metabolism of the iron bacteria has been under discussion for many years. (Winogradsky (1881), Molisch (1910), Cholodny 1926). Molisch considers the presence of iron in the Iron bacteria as accidental. Molisch himself however found, that the iron favours intensely the development of the iron bacteria. He also found that the iron can be replaced by manganese. This was surprising to him, because otherwise manganese cannot replace iron in the plant metabolism. This fact is one of the reasons which leads Molisch to believe, that there is no connection between the deposit of iron and the metabolism of the iron bacteria. I think, that there is another important fact which explains these divergences: iron is a very intense catalyzer for oxygen. In this quality it can be replaced by manganese. (Pribram, unpublished experiments.) Considering this fact we arrive at the conclusion, that the iron plays a similar rôle in the metabolism of iron bacteria to that played by Chlorophyll in the metabolism of higher plants. For this reason we treat the iron containing Schizomycetes together, forming the order Siderobacteriales. The first family of this order are the *Chlamydotrichaceae*. The name "Chlamydobacteriaceae" used in Bergey's Manual "does not conform to the nomenclatural rule, that the name of a family should be fashioned from one of the constituent genera" (Buchanan, l.c.p. 251). The name Chlamydotrichaceae which we suggest for this family is taken from the genus Chlamydothrix. This name has been proposed by Migula (1900) and has been widely used for the genus Leptothrix (Kuetzing). On account of the similarity of the names Leptothrix and Leptotrichia (Trevisan) of the subclass Mycobacteria the name Chlamydothrix may be proposed for the genus of the order Siderobacteriales.

The forms of the family Chlamydotrichaceae are: filamentous cells with iron deposits in their sheaths. Tribe *Chlamydotricheae*, unbranched filaments, has two genera: the *Leptothrix* (*Chlamydothrix Migula*) the filaments of which are not attached and the *Crenothrix* with attached filaments. Tribe 2 are the branched filaments, the *Clonotricheae*, which have one genus, the *Clonothrix*. Lehmann (l.c.p. 521) calls our attention to the fact, that the name Clonothrix has been used by Roze for a blue Alga (1896). Cholodny does not differentiate between Clonothrix and Crenothrix. The second family of the order Siderobacteriales is the family *Siderocapsaceae*. This family is characterized by short cells. The first genus, *Didymohelix*, had to be moved from the first to the second family (tabulation). Bergey following Buchanan's classification describes these organisms as filaments. The filaments however are excretions, which contain iron (Cholodny 1926). The body itself is a short vibrio-like cell, 1, 2 to 1, 4 micra in length, and 0, 5 to 0, 6 micra in diameter. We treat them therefore with the two following genera. The two forms, *Siderocapsa* and *Sideromonas*, are not mentioned in Bergey's Manual. Siderocapsa has short rods or cocci enclosed in a zoogloea surrounded by a large area which contains iron. The genus Sideromonas includes cocci or short rods forming chains. These chains are enclosed in a dense capsule which contains iron.

The third order of the subclass Algobacteria, the order *Thiobacteriales*, is characterized by the deposit of sulphur or bacteriopurpurin. The first family the *Rhodobacteriaceae*, contains forms with bacteriopurpurin, the second family, the *Beggiatoaceae*, are filamentous non-motile forms with sulphur. The third family, the *Achromatiaceae* are motile not filamentous cells containing sulphur granules. The subfamilies of the family Rhodobacteriaceae are the *Chromatoideae* with sulphur granules and bacteriopurpurin and the *Rhodobacteroideae* with bacteriopurpurin but without sulphur granules. As we follow in this order exactly the classification of Buchanan we refer for details (enumeration of tribes and genera and their characteristics) to our tabulation.

The fourth order of the Algobacteria are the *Myxobacteriales*. They produce a gelatinous aggregation, then they become en-

cysted. The first family, the *Polyangiaceae* are rods, which are shorter, before they become encysted, but are not transformed into coccus like spores. The genus *Chondromyces* of this family forms free cysts, the genus *Polyangium* forms cysts in a gelatinous mass. The second family of the order Myxobacteriales, the *Myxococcaceae* are rods, which are transformed into coccus like spores before they become encysted. There is ony one genus, the *Myxococcus*.

Summary

Suggestions regarding the classification of the Committee of the Society of American Bacteriologists have been offered according to the following principles: selecting the common characteristics of coördinated groups in order to hereby obtain a definite terminology; employing only one characteristic or a few common characteristics for one group and making subdivisions for further characteristics, securing thus a higher adaptability and elasticity; assigning the same characteristics as the fundamental bases for coördinated groups in order to render the survey as comprehensive and easy as possible.

According to the method used by the Committee those characteristics were given first place which are biological characteristics. Furthermore, we tried to trace those characteristics which are connected with the metabolism of the microörganisms in order to use them as fundamental principles for higher groups.

Because of their phylogenetic relationship to other classes four subclasses were formed: Protozoobacteria, Eubacteria, Mycobacteria and Algobacteria. The subdivisions can easily be understood from our tabulation.

A study of the classification revealed that the Schizomycetes have the following characteristics due to the nature of the medium on which, or in which, they live. This is evidence of an adaptation to a certain medium.

There is a tendency to act upon inorganic compounds in the family Protobacteriales and a tendency to utilize organic compounds in the family Metabacteriales. Those forms which are adapted to the soil and water and to plants have plant char-

acteristics. They form ramified, arborescent or curled colonies. They form mycodermata on the surface d liquid media. Other families form long threads or the cell body itself is ramified. Production of granules of a higher resistance (Pfeifferella, Pasteurella, Lactobacillus, Corynebacterium), spore-formation, development of a high resistance of the whole body such as Gram resistance also acidoresistance and even production of cellulose (*Acetobacter xylinus*) can also be considered as plant characteristics. Other plant characteristics are: growth under anaerobic conditions, lack of motility, budding, such as is present in proliferation by means of spores and in ramification, production of water insoluble pigments, deposit of iron and of sulphur and in higher forms of chlorophyll utilizing light for metabolism.

The accommodation to the semiliquid organism of animals can be demonstrated by: formation of spherical colonies, imitating the spherical shape of all animal organs, formation of short, to a greater extent of motile forms, as well as proliferation exclusively by fission, never by any process which is connected with budding. Relatively lower resistance of the body, characterized by the negative Gram staining corresponds to the softer structure of the animal body.

All forms are connected by connecting links which show characteristics of different more or less related groups.

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A COMPARATIVE STUDY OF DENTAL ACIDURIC ORGANISMS AND LACTOBACILLUS ACIDOPHILUS

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It is now generally recognized that certain aciduric bacteria play an important part in the production of dental caries. On the basis of their morphological and cultural characteristics, these organisms have been classed with *Lactobacillus acidophilus* of the intestine; but, of the extensive literature relating to the bacteria of dental caries, on the one hand, and to the intestinal aciduric bacteria, on the other, only a few scattered and rather inconclusive papers deal directly with a comparison of the two groups. In 1924, Hilgers, on the basis of a briefly reported serological comparison, concluded that *B. necrodentalis* of Goadby, (1903) isolated from teeth, is a slight variant of *L. acidophilus*, and is the equivalent of the "*B. acidophilus-odontolyticus*" which McIntosh, James, and Lazarus-Barlow had isolated and studied in 1922. In 1924, McIntosh, James, and Lazarus-Barlow recorded cross-agglutination of known strains of *L. acidophilus* with the antiserum of a dental strain. In 1927, Ross, Krasnow, and Samet, comparing a milk strain of *L. acidophilus* with a dental aciduric strain, found them serologically related. In contrast to the foregoing, Morishita (1928, 1929) believes that marked differences exist between the dental and intestinal strains. In two preliminary papers, which, like that of Hilgers, unfortunately omit full details, he has reported the results of extensive work on the question of the relationship of *L. acidophilus*

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to two of five groups of dental aciduric organisms. Morishita concludes, in regard to the first of his groups, that "the tooth organisms . . . . constitute a type entirely separate from *B. acidophilus* of the intestinal tract," and similarly finds no close relation between *L. acidophilus* and his second group.

The present report deals with an attempt to resolve the confusion indicated by the literature. To this end we have conducted an intensive study of a group of aciduric bacteria isolated from dental caries, in conjuncton with a group of known strains of *L. acidophilus*, under strictly comparative conditions.

SOURCE OF STRAINS EMPLOYED

The dental strains employed in this work were obtained in the clinics of the School of Dental and Oral Surgery of Columbia University[2] at the Medical Center, from the mouths of an unselected group of clinic patients, ranging in age from 8 to 66 years. The method of isolation employed, based on that used by McIntosh, James, and Lazarus-Barlow (1922), Rodriguez (1922), and Bunting, *et al* (1925, 1926) was as follows: Scrapings taken from cavities and interdental spaces with a sterile nichrome excavator, (made for the purpose from a heavy nichrome needle) were inoculated into 1 per cent glucose infusion broth, pH 5.0, and incubated from 48 to 72 hours at 37°C. Gram stains were then examined, and all cultures showing Gram-positive rods were streaked on casein digest agar, prepared according to the formula of Hunter (1924). After incubation for 48 hours at 37°C., typical faint, punctiform colonies, if these had appeared, were transferred back into pH 5.0 broth. In many instances the first plate showed only the typical colonies; in others the growth was mixed, and it was necessary to replate three or four times before the contaminating forms (yeasts, almost exclusively) were eliminated. In still other cases repeated attempts to isolate the typical bacilli were unsuccessful, and these cultures were eventually abandoned. The cultures, when pure, were trans-

ferred to casein digest broth, reaction unadjusted (about pH 7.4), in which they were kept growing by subculturing every few days. The purity of the cultures was confirmed at intervals throughout the work, by Gram strains, and by plating.

In addition to the cultures which had to be abandoned because of the persistent overgrowth of other mouth organisms, (yeasts, chiefly) in a number of instances the typical aciduric bacilli were not obtained at all. This circumstance is ascribable in part to errors in technique at the beginning of the work, and also, in all probability, to the considerations mentioned by Bunting, *et al.* (1928) in this connection.

The following strains of known *L. acidophilus* were employed. The first seven strains listed were given to us through the courtesy of Dr. N. Kopeloff, of the New York State Psychiatric Institute and Hospital, labeled with the sources indicated:

    I. (Ac49)   W. R. Albus, Dairy Div. U. S. Dept. Agriculture
    II. (1482E)   H. K. Mulford Co. (Y strain)
    III. (Weir Y)   Y strain recovered after feeding patient 1482E
    IV. (1482 Gretsch)   Y strain recovered after feeding patient 1482E
    V. (1482A)   H. K. Mulford Co. (Y strain)
    VI. (Torrey M)   Dr. J. C. Torrey, Cornell University
    VII. (KI)   Dr. N. Kopeloff
    VIII. S. L. Leiboff, Columbia University (Kopeloff's strain)
    IX. Dr. J. A. Anderson, Rutgers College.

MORPHOLOGICAL AND BIOCHEMICAL STUDY—METHOD

The 21 strains of dental aciduric organisms which had been isolated in pure culture, and the 9 known strains of *L. acidophilus*, were kept growing uniformly in casein digest broth throughout the course of the work. In the morphological and biochemical studies, each of the various tests employed was conducted with all of the thirty strains under the same conditions, with occasional exceptions as indicated.

The morphological data were obtained from an examination of Gram stains of cultures growing in casein digest broth at 37°C. for 24 hours, 48 hours, and 10 days, and of colonies on streaked casein digest agar plates growing at 37°C. for 48 hours and 10 days. Colony form was studied in 1 per cent glucose agar pour

plates, inoculated with one loopful of casein digest broth culture 48 hours old, and incubated for 48 hours at 37°C. The biochemical study consisted of fermentation tests on nine carbohydrates, action on litmus milk, and acid production in broth cultures. Fermentation studies were made in broth freed of sugar by *B. coli* inoculation, and containing 1 per cent of the fermentable test substance. In the case of raffinose, salicin, mannitol, trehalose, and sucrose, the absence of reducing sugars was determined in the sterile fermentation tubes by Benedict's reaction, which was negative in each case. The fermentation tubes were each inoculated with three loopfuls of a young culture in casein digest broth, and incubated at 37°C. for 72 hours. The presence or absence of acidity was then tested by adding 2 drops of Andrade indicator to each tube. The results with each test substance were compared with two controls: an uninoculated tube of the test substance incubated with the fermentation tubes, and a tube to which three loopfuls of an old, strongly acid culture were added immediately before adding Andrade indicator, the latter to test the limit of acidity imparted to the fermentation tube by the broth in the inoculum. All negative results were checked at least once. In these fermentation studies no attempt was made to determine the degree of acidity produced, beyond the mere presence or absence of acidity, definitely indicated. The degree of acidity produced by the strains was determined separately, as shown below.

Studies in litmus milk comprised two series of tests: one, on a selected group of nine strains (five dental, four intestinal) inoculated with 1 cc. of broth culture and observed in the incubator at two-hour intervals for twelve hours; the other, including all thirty strains, inoculated with 3 loopfuls of broth culture, incubated at 37°C., and examined daily for one week, and at weekly intervals four times thereafter. With each series a control tube of uninoculated litmus milk was incubated and examined during the same period.

The extent of acid production by the thirty strains was determined in cultures in casein digest broth (approximate pH 7.4) which had been incubated for two weeks or more. The Gillespie

drop-ratio method (Clark, 1928) was employed, using brom phenol blue in 5 cc. standards.   Each acid standard contained one cc. of sterile broth for color compensation.   The determinations were made in tubes containing 1 cc. of the culture, diluted with 4 cc. of distilled water.   The standard tubes were prepared and used on the same day.

MORPHOLOGICAL STUDY

All the organisms studied, including both the dental and the intestinal groups, were characteristically rather slow growing forms.   The dental strains, in the acid broth employed during the period of their isolation, showed little or no growth in 24 hours at 37°C., and poor to moderate growth in 48 hours.   Casein digest broth, which is richly nutrient, produced better growth; but even in this broth it was necessary to inoculate heavily.   Transplantation of 1 cc. with sterile pipettes gave the best results.   Several strains, among both the dental and the intestinal groups, showed at intervals a tendency to die out; most of these were revived satisfactorily by transplanting them repeatedly through casein digest broth at 24 to 48 hour intervals, but three of the dental strains (nos. 27, 33, 49) remained weak.   The other strains, after repeated subculturing, grew well in casein digest broth in 24 hours.   Growth on agar was similarly meager.   On both glucose agar and casein digest agar little or no growth was observed in 24 hours, and in 48 hours colonies varied from about one millimeter in diameter down to macroscopic invisibility.   Colonies on casein digest agar were larger than on glucose agar.

The general appearance of growth in broth, mentioned by Morishita (1928) as a distinguishing characteristic between the dental organisms and *L. acidophilus*, was not generally different in our two groups.   Variation in appearance seemed to depend more on the age and extent of the growth than on any intrinsic differences in growth tendency.   Morishita reported that his dental group showed uniform turbidity in broth, whereas *L. acidophilus* tended to grow as sediment at the bottom of the tube. Our thirty strains, observed on nine different occasions, (including six series of cultures) indicated the following: All of the strains of

both groups showed uniform turbidity in the broth at the first appearance of growth, with very slight sedimentation or none at all.   Two of the dental strains (nos. 27 and 33, both persistently poor growers) showed uniform turbidity during 48 hours' incubation.   Of the remainder, 10 dental strains and 5 intestinal strains tended to sediment completely within 48 hours, the supernatant broth becoming clear, or showing scattered floccules; while 9 dental strains and 4 intestinal strains sedimented incompletely, the broth remaining turbid.

An intensive morphological study of the thirty strains, in which Gram preparations of each strain were studied carefully from broth and agar and their characteristics recorded, also failed to reveal any consistent difference between the mouth organisms and *L. acidophilus.*   All thirty strains were pleomorphic, some more so than others; in general the dental strains were found variable within wider limits than the intestinal strains, but the direction and kind of variation were the same among both groups.

All of the strains showed, as the predominating form, Gram-positive rods with rounded ends, occurring singly, in chains, and in palisaded clumps.   The mean dimensions of the rod were 0.5 by $2-5\mu$; the extreme variations recorded were, in width, from 0.3 to 1.0 μ, and in length, from 0.6 to 10.0 μ, exclusive of filamentous forms.   While in the majority of instances individual cultures showed rods of fairly constant width, each strain varied more or less widely in this dimension at different times; and several strains of both groups (seven dental, two intestinal) occasionally showed both thick and thin rods in the same culture.   One strain (no. 4, dental) appeared consistently as a thin rod; 8 dental and 2 intestinal strains never exceeded a width of 0.6μ; 9 dental and 7 intestinal strains varied in width from 0.3 to 0.8 μ, and 3 dental strains varied from 0.3 to 1.0 μ.

In 24 hour casein digest broth cultures, both groups showed a tendency to grow in long chains, the chains becoming shorter from 24 to 48 hours, with the appearance of palisaded clumps. On young (48 hour) casein digest agar, both groups occurred almost entirely as chain pairs and palisaded clumps, with an occasional short chain.

In addition to the predominating rod form, four types of variants were observed: (*a*) coccoid forms, occurring as single round or ovoid forms or as streptococci, the latter often with a few rod forms in the chain; (*b*) beaded rods, as united diplococcal forms, as diphtheroids with Gram-positive poles and a Gram-negative center, or as irregularly curved, beaded rods having the form of tubercle bacilli;[3] (*c*) as curved rods, occurring singly, in ring pairs, or, occasionally, in short, irregular curved chains; and (*d*) as short or moderately long unbranched filaments. All four variant forms were seen at different times in only one dental strain (no. 38) and one strain of *L. acidophilus* (no. I). Eleven dental and six intestinal strains showed three of the four forms, five dental and one intestinal strains showed two, and four dental and one intestinal strains showed one. Coccal forms occurred in 11 dental strains, 2 intestinal strains; beaded forms in 13 dental strains, 6 intestinal strains; curved forms in 14 dental and 8 intestinal strains, and filaments in 13 dental and all 9 intestinal strains.

In young broth cultures (24 to 48 hours old) the dental group showed all four variant forms, though infrequently; coccal and beaded forms were not seen among the strains of the intestinal group in young broth. In 48 hour agar, all the variant forms occurred in both groups; filaments predominated among the dental strains, curved forms among the intestinal. In old broth cultures variant forms were seen in greater profusion: beaded forms predominated among both groups. In old agar cultures beaded forms were not seen; curved forms occurred only in two instances, both in intestinal strains; coccal forms and filaments occurred commonly.

All of the forms described here, with the exception noted,[3] have been previously reported by Howe and Hatch (1917), McIntosh, James, and Lazarus-Barlow (1922), Rodriguez (1922), and Bunting and Palmerlee (1925).

In the matter of colony form, as in other respects, although a considerable variation was encountered among the thirty strains,

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[3] These forms were seen only in milk cultures incubated 34 days at 37°C.; they have not, so far as we know, been previously described.

both groups of organisms were found to vary around a common mean; and no basis of differentiation could be made out from the recorded data.   Morishita (1928, 1929) speaks of the colony forms of *L. acidophilus* and his dental groups as "markedly different"; he does not report having seen the rough or "X" type colony among his dental strains.   The results of a careful study of our thirty strains in this respect follow:

Colonies were found to be larger on casein digest agar than on glucose agar; but the latter medium was used for the comparative studies because the granularity of the casein digest agar interfered with careful study of colony form.

Colonies growing on the free surface of glucose agar pour plates were relatively few in number, as compared with the invariably extensive subsurface growth.   The surface colonies were puncti-form and very small, often barely visible to the naked eye.   Under the low power of the microscope all surface colonies observed were pale, translucent, faintly yellowish, and finely granular by trans-mitted light.   Adjoining colonies were confluent.   Colony out-lines varied from perfectly circular, with entire margin, to irregu-lar, with margins erose and somewhat lacerate.

Subsurface colonies were numerous in all cases, and smaller than the surface colonies, giving to the plate, when examined by transmitted light with the naked eye, an appearance of fine, even granularity.   Under the low power three types of colonies were distinguished: (*a*) the smooth or so-called "Y" type—round, elliptical, fusiform, or irregular in outline, slightly translucent, finely granular, brownish; margin entire, sharply defined, smooth, or with occasional lobulations; (*b*) the rough or so-called "X" type—irregular in shape, margin generally and very irregularly filamentous, the filaments being tangled and projecting from a brownish central core, the filaments sometimes observed to be longer at one or two poles; (*c*) intermediate forms, presenting a smooth, sharply defined margin extending around part or all of the periphery, with few filaments all around, or a tuft only at one or two poles.

Variations in colony form among the individual strains may be summarized as follows, on the basis of two series of comparative

platings: Ten dental strains and eight intestinal strains showed surface colonies with regular margins in both instances; two dental strains showed surface colonies with irregular margins in both instances; the remaining nine dental strains and one intestinal strain (no. I) were variable, showing both types of margins. One dental strain (no. 35) showed only the "X" type of deep colony. One dental strain (no. 34) and one intestinal strain (no. IV) showed only the "Y" type. Three dental strains showed only the "X" type in one plating, and only the "Y" type in the other. The remaining 16 dental strains and 8 intestinal strains showed all three forms of deep colonies.

The colony forms described here agree generally with those reported among the dental aciduric bacteria by McIntosh, James, and Lazarus-Barlow (1922), and by Bunting and Palmerlee (1925).

The colony form of individual strains was not consistent, in most cases, in the two platings; although the intestinal strains showed very much more uniformity in this respect than the dental strains. Fourteen dental and eight intestinal strains appeared similar in the two platings, with some variation occurring in the relative preponderance of types; the other seven dental strains and one intestinal strain (no. IX) were markedly variable, showing forms in one plating which were not seen in the other. This agrees with the finding of Kulp and Rettger (1924), who have reported that the colony form of *L. acidophilus* is not stable.

BIOCHEMICAL STUDY

In the comparative fermentation studies, the two groups of strains reacted similarly with the substances tested. In general, the two groups showed a similar marked ability to ferment glucose, lactose, maltose, levulose, and trehalose, and a common inability to ferment raffinose. No gas was produced by any of the strains. The details of the results of these tests may be summarized as follows:

All thirty strains fermented glucose, lactose, and levulose.

All but one strain (no. 49, dental) fermented trehalose. Raffinose was not fermented by any of the strains.

The results with maltose, salicin, and mannitol were somewhat variable. Two dental strains (nos. 4 and 18) and one intestinal strain (no. III) failed to ferment maltose in one of three series of tests, fermenting it in the other two. The remaining strains of both groups fermented maltose consistently. Salicin, when first tested, was fermented by all but two dental strains (nos. 38 and 49), and by only four intestinal strains, and mannitol by all but the same two dental strains, and all but three intestinal strains. On repeating the negative results, all of the strains except no. 49, dental, (which was consistently negative) fermented both salicin and mannitol.

Sucrose gave markedly variable results. Tests made soon after isolation, in the case of the dental strains, and soon after we had obtained them, in the case of the intestinal strains, indicated that only four dental strains and one intestinal strain (no. I) were able to ferment this sugar. All of the negative results were repeated, and were again negative. However, after three months, during which time all of the strains had been growing uniformly, all thirty strains were able to ferment sucrose. Since all factors within control were the same in both series of tests, the difference in these results would seem to depend on inherent differences in the organisms themselves during the two periods. The circumstance that all of the strains were growing more vigorously at the time of the second series of tests may account for the discrepancy. McIntosh, James, and Lazarus-Barlow (1924) have reported, with regard to their *L. acidophilus-odon-tolyticus*, that non-fermenters of sucrose may acquire the ability to ferment it after prolonged subculturing.

All of the strains studied acidified and coagulated litmus milk; but a very considerable variation was observed in the length of time required by the strains to produce this result, especially among the dental strains. In the series of milk tubes inoculated with three loopfuls of broth culture, sixteen dental and eight intestinal strains coagulated the milk in from 48 to 96 hours. Of the remainder, one dental strain produced coagulation in five

days, one dental and one intestinal strain in six; one dental strain required seven. The other two dental strains coagulated the milk only after the lapse of 14 days in one case (no. 49), and 34 days in the other (no. 33); all were incubated continuously at 37°C. The uninoculated control tube, incubated during the same period, remained unchanged. These time requirements are apparently relative, and seem to depend chiefly, if not entirely, on the rate of growth of the individual strain, and the amount of inoculum. In the selected series inoculated with 1 cc. of broth culture, all of the tubes (including no. 49) showed complete coagulation within 12 hours at 37°C. Consequently, the authors do not believe that the relatively longer time required by some of the dental strains for the coagulation of milk can be used to differentiate them from L. acidophilus.

The similarity of the two groups in this relation was indicated clearly by a study of the stages of the process of coagulation. In this study, the selected group mentioned above, inoculated with 1 cc. of broth culture, was employed to study the initial stages; the complete group, inoculated with 3 loopfuls of broth culture, was used to observe the later stages.

On the basis of a difference in the early stages of coagulation, the thirty strains may be divided into two types, designated for convenience A and B. Type A includes 18 of the dental strains and 5 of the intestinal. Type B includes 3 dental and 4 intestinal strains.

The process of coagulation in the first group (A) was found to be as follows: The first change (observed, during continuous incubation at 37°C., in the tubes containing 1 cc. of inoculum, in 3 to 6 hours) consisted of a progressive acidification, followed by decolorization, of the litmus, the lower portion of the tube preceding the upper; coagulation occurred after the lower portion of the tube had become completely decolorized. In the second group (B) the change was gradual and uniform, the tube becoming progressively more acid until coagulation ensued. After coagulation was complete, decolorization of the litmus began at the bottom of the tube and extended upward. From this point on, the tubes of both types had the same appearance. The coagulum

was hard; a small amount of whey ran out of it on tipping the tube. A completely decolorized zone, in the lower portion of the tube, was marked off sharply from the pink zone above. The decolorized zone varied in extent, apparently according to the amount of growth present. After this point, during continued incubation, when the decolorized zone had attained a maximum extent, it was progressively reduced in size, and eventually disappeared entirely, leaving the coagulum uniformly pink. It is interesting to note that, in smears made from six of the tubes which had been incubated for 34 days, and in which the coagulum had shrunk to one-third of its original bulk, typical organisms were found in each case, many showing marked beading.[4]

Notably similar results were also obtained in the matter of acid production by the thirty strains, if the exceptions provided by the three consistently slow growing dental strains be discounted. The limit of acidity produced by the intestinal strains varied from pH 3.5 to pH 3.7; that produced by the dental strains, excepting nos. 27, 33, and 49, between pH 3.5 and pH 3.8. A pH of 4.0 was the limit attained by strains 27 and 33, while no. 49 failed to produce acidity beyond pH 4.1.

AGGLUTINATION REACTIONS—METHOD

In order to study the immunological relationships of the dental and intestinal groups, sera were prepared against one of the known *L. acidophilus* strains (no. VI) and against one of the mouth organisms (no. 4). Both of these strains were selected at random from among the more vigorous growers.

The following were the methods employed in obtaining the sera: In order to secure heavy growth, cultures were transferred through casein digest broth at 24 hour intervals for several days, using 1 cc. of inoculum. A 24 hour culture of each strain was then centrifuged, and the organisms resuspended in physiological saline. Each cubic centimeter of this suspension contained approximately the number of organisms in 10 to 15 cc. of the original broth culture. One cubic centimeter of each suspension

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[4] See footnote on page 401.

thus prepared, containing living organisms, was injected three times a week intraperitoneally into rabbits. After seven injections of the mouth organism and eight of the *L. acidophilus*, the sera were tested against their homologous strains and found to have titers of 1:1000 and 1:500 respectively. As these titers were considered satisfactory, the animals were exsanguinated and the sera used as described below.

The agglutinations were carried out as follows: Cultures were grown in casein digest broth, centrifuged, and resuspended in saline as before. One-half cubic centimeter of this suspension was mixed with 0.5 cc. of the various serum dilutions. The control tubes contained 0.5 cc. of the suspension and 0.5 cc. of normal saline. The tubes were placed in the water bath at 37°C. for one hour, read, and then left in the ice box until the next day, when they were read again. In many cases readings were also made after 48 hours, but none of these showed any changes from the 24 hour readings.

AGGLUTINATION REACTIONS—RESULTS

The results of the agglutination tests are given in the table. Two of the dental strains (nos. 10 and 49) agglutinated spontaneously throughout. Two other dental strains showed spontaneous agglutination, one (no. 15) with the antiserum to the intestinal strain, the other (no. 33) with the antiserum to the dental strain. This difficulty was not encountered among the intestinal strains. Of the two strains used for preparing the sera, the dental strain (no. 4) agglutinated with its own serum up to a dilution of 1:1000, and with the "intestinal' serum up to 1:500. The intestinal strain (no. VI) agglutinated with its own serum to a dilution of 1:500, and with the "dental" serum to 1:100.

Inspection of the table shows further that 12 of the dental strains agglutinated with the no. VI serum to a dilution as high as or higher than with the no. 4 serum. Conversely, 5 intestinal strains agglutinated with the no. 4 serum to a dilution as high as or higher than with the no. VI serum.

Serologically there is no evidence of separate groups within the dental strains (discounting those which agglutinated spontaneously) except in the case of strain no. 25, which agglutinated

TABLE 1

Dental

STRAIN	SIZE LIMITS OF ROD Width μ	SIZE LIMITS OF ROD Length μ	VARIANT FORMS Coccal	Beaded	Curved	Filam.	GROWTH IN BROTH	COLONY FORM Surface	Deep	REMARKS	FERMENTATION Glucose	Lactose	Maltose	Sucrose	Levulose	Trehalose	Raffinose	Salicin	Mannitol	LITMUS MILK Coagulation time days	Type	LOWEST ACIDITY pH	AGGLUTINATION HIGHEST TITER + (1:) Serum 4	Serum VI
4	0.3–0.4	1–6	0	+	+	+	U-S	Sm	Y–X, v		+	+	∓	∓	+	+	0	+	+	2	A	3.6	1000	500
8	0.3–0.5	1–4	+	+	+	0	U-S	Sm	Yx		+	+	+	∓	+	+	0	+	+	3	A	3.5	1000	750
9	0.3–0.8	0.8–6	+	+	+	0	I	SR	XY		+	+	+	∓	+	+	0	+	+	4	A	3.7	1000	750
10	0.3–0.7	0.8–6	+	0	+	0	I-S	RS	XY		+	+	+	∓	+	+	0	+	+	4	A	3.5	S.A.	S.A.
15	0.3–0.8	1–10	0	+	0	+	U-I	Sm	Xv		+	+	+	+	+	+	0	+	+	3	A	3.7	1000	S.A.
18	0.4–0.7	0.6–5	+	0	+	0	U-S	Sm	XY	Coccoid forms predominated in broth at one period	+	+	+	+	+	+	0	+	+	5	A	3.5	1000	500
20	0.3–0.9	0.8–6	+	+	+	0	U-I	Sm	Yx, v		+	+	+	+	+	+	0	+	+	4	A	3.5	200	750
21	0.3–0.7	1.5–6	0	+	+	+	U-I	Sm	Xy, v		+	+	+	∓	+	+	0	+	+	2	B	3.6	100	100
22	0.3–0.7	1–7	+	+	+	+	U-I	Sm	X–Y, v		+	+	+	∓	+	+	0	+	+	2	A	3.7	100	250
25	0.3–0.8	1–3	+	+	0	+	U-I	Sm	X–Y, v	Isolated from a true immune	+	+	+	∓	+	+	0	+	+	2	B	3.5	50	0
27	0.3–0.8	1–6	+	0	0	+	U	SR	Xy	Consistently slow grower	+	+	+	∓	+	+	0	+	+	7	A	4.0	100	50
29	0.3–0.7	1–7	0	0	0	+	U-I	SR	Xy	⎱ Tendency to die out overcome by frequent successive transplants	+	+	+	∓	+	+	0	+	+	3	A	3.8	200	500
31	0.4–0.6	1.5–10	0	0	0	+	U-I	Sm	Xy, v	⎰ Consistently slow grower	+	+	+	∓	+	+	0	+	+	6	B	3.8	200	50
33	0.3–0.7	1–7	0	0	+	+	U	SR	XY		+	+	+	∓	+	+	0	+	+	34	A	4.0	S.A.	100
34	0.3–0.5	2–6	0	0	0	+	I-S	Sm	Y		+	+	+	∓	+	+	0	+	+	3	A	3.7	500	500
35	0.5–0.6	1.5–6	0	+	0	+	U-S	R	X		+	+	+	∓	+	+	0	+	+	3	A	3.7	500	500
38	0.3–1.0	0.6–6	+	+	+	+	U-S	SR	XY	Showed very thick rods only in broth at one period	+	+	+	+	+	+	0	∓	∓	2	A	3.7	0	250

	No.																						
Dental	39	0.3–0.8	0.7–6	+	+	+	0	U-S	SR	XY	Tendency to die out overcome by frequent successive transplants	+	+	+	±	+	+	0	3	A	3 6	1000	750
	41	0.4–0.8	8–6	+	+	+	0	U-S	RS	Xy, v		+	+	+	±	+	+	0	4	A	3 5	200	250
	47	0–1.0	8–6	+	+	+	0	U-S	SR	Xy		+	+	+	±	+	+	0	4	A	3 7	100	100
	49	0.4–0.7	1–8	0	+	0	+	U-I	R	Yx	Consistently slow grower	+	+	+	±	0	0	0	14	B	4 1	S.A.	S.A.
Intestinal	I	0.3–0.8	1–6	+	+	+	+	U-I	RS	Xy		+	+	+	±	+	+	0	4	B	3 5	0	500
	II	0.4–0.6	8–6	0	+	0	+	I-S	Sm	Yx		+	+	+	±	+	±	0	4	B	3 5	100	100
	III	0–0.8	8–6	+	0	+	+	I-S	Sm	XY		+	+	±	±	+	±	0	4	B	3 6	200	500
	IV	0.3–0.7	1–8	0	+	0	+	U-S	Sm	Y		+	+	+	±	+	±	0	2	A	3 7	500	500
	V	0.4–0.7	8–6	0	+	0	+	U-I	Sm	Yx		+	+	+	±	+	±	0	2	A	3 6	100	250
	VI	0.3–0.6	6–6	0	+	0	+	U-I	Sm	XY		+	+	+	±	+	+	0	2	A	3 6	100	500
	VII	0.5–0.8	1–10	0	0	+	+	U-S	Sm	Yx	Tendency to die out overcome by frequent successive transplants	+	+	+	±	+	+	0	2	A	3 5	1000	500
	VIII	0.5–0.8	2–7	0	+	+	+	U-S	Sm	XY		+	+	+	±	+	±	0	6	A	3 7	500	500
	IX	0.5–0.6	1–6	+	+	+	+	U-I	Sm	Xy, v		+	+	±	±	+	±	0	4	B	3 5	1000	100

Key to tables: U, uniform turbidity; S, complete sedimentation; I, sedimentation, broth remaining turbid; Sm, smooth (having regular margin); R, rough (having irregular margin); SR, showing smooth and rough forms at different times; RS, showing smooth and rough forms on the same plate; X–Y, varying from pure "X" type to pure "Y" type; Yx, Xy, showing predominance of the first form, respectively; XY, showing both forms without predominance; v, variable; A, decolorization before coagulation; B, coagulation before decolorization; S.A., spontaneous agglutination.

with the no. 4 ("dental") serum only, and only at the lowest titer used.   Strain no. 38 was also peculiar in that it agglutinated with the no. VI ("intestinal") serum up to 1:250, but did not react at all with the no. 4 serum.   Strain no. I presents the only instance of agglutination to a high titer with the serum of the homologous group, and complete absence of cross-agglutination.

Of the strains not included above, nos. 18, 27, and 31 showed only slightly greater agglutination with the no. 4 serum than with the no. VI serum; nos. III and V showed agglutination at rather higher titers with the no. VI serum than with the no. 4 serum. On the whole there is indication of a slightly greater homogeneity in the intestinal group than in the dental group.   This is consistent with the morphological and biochemical results obtained with these strains, and is perhaps to be expected from the conditions under which the two groups were obtained.

The results obtained in this work with agglutination reactions confirm those of Hilgers (1924), but are in apparent disagreement with the findings of Morishita (1928, 1929).   The serological homogeneity of the dental organisms has been shown by McIntosh, James, and Lazarus-Barlow (1922), and confirmed by Sierakowski and Zajdel (1924).

<center>DISCUSSION</center>

A consideration of all the data pertaining to the individual strains, in their morphological, biochemical, and serological relationships, does not disclose any individual differences consistent enough to indicate lack of close relationship.   The more important features of each of the thirty strains are included in the table.   The table indicates clearly that, in all their relations, most of the dental strains, and most of the intestinal strains, belong to a single group within which there is no evidence for subdivision.   The absence of serological data relating to strains 10 and 49 is unfortunate.   In its other relations, strain 10 is typical.   Strains 27, 33, and 49 were consistently slow growers. These three strains, as shown in the table, gave the most aberrant results in milk and in acid production; no. 49 was especially atypical in its fermentative powers.   Whether these differences are intrinsic in the strains, or result merely from an inability of the

organisms, not shared by the other strains, to adapt themselves to artificial conditions, the authors are not prepared to say. Further evidence relating to these strains might throw the balance either way.

The failure of strain 38 to agglutinate with the serum of the dental group in any dilution allies this strain with no. I (intestinal), which reacted similarly in this respect.   These two strains are also similar in other respects.   Both were active growers; they were the only strains which showed all four of the variant forms, and both consistently fermented sucrose.

Strain 25 presents an especially interesting variation in its failure to agglutinate with the serum of the intestinal group in the dilutions used, and its low titer with that of the dental group. Although in other respects it is not atypical, aside from the circumstance that the rods never exceeded a length of 3.0 μ, it was found, on looking back over the records of cases from which the dental strains were isolated, that this strain was the only one obtained from an apparently true caries-immune mouth (Miss E. R., aged 18)—presenting a full complement of teeth, with no fillings, and no evidence of caries.   This circumstance is presented as an interesting coincidence of exceptional relations; its significance is not understood.

SUMMARY

Twenty-one strains of aciduric organisms isolated from teeth, and nine strains of L. acidophilus obtained from various sources, have been studied comparatively in their morphological, biochemical, and serological relations.   The results of this study are as follows:

No consistent difference could be discovered between the groups in any part of the morphological or biochemical study. All of the strains studied were generally variable, the dental group somewhat more so than the intestinal; but these differences were in degree rather than in kind, and were slight at most.

In their serological reactions, no clear distinction between the two groups could be found.   Most of the strains of both groups showed marked cross-agglutination.   Only one dental strain was distinctly aberrant in this respect.

CONCLUSION

While the authors do not, of course, feel justified, on the basis of a study comprising only thirty strains, in generalizing to include all dental aciduric bacteria, they see no reason at present, pending a more extensive investigation of the subject, for differentiation between these organisms and *L. acidophilus* of the intestine.

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GRAM STRUCTURE OF COCCI

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The fact that changes similar to those which exposure to acriviolet produces in *B. anthracis* and allied organisms, could not be brought about when cocci and other Gram-positive organisms were thus treated, led to the assumption in previous reports on this subject (Churchman, 1927a and 1927b) that the Grampositivity of the two types of organism depended on a different mechanism.

It has since been found, however, that the kind of changes reported for *B. anthracis* can be produced by other means in certain species of cocci; and that these organisms, too, probably possess a Gram-positive cortex and a Gram-negative medulla. Studies with the yeasts have been less satisfactory than those with the cocci and the results have not been sufficiently constant to warrant positive conclusions. A good deal of evidence has been accumulated, however, for the belief that the Gram structure of the yeasts is similar to that of *B. anthracis* and the cocci. It should be recalled, in this connection, that Breinl (1923) has shown that certain strains of staphylococci were changed, by what he regarded as artificial mutations, into Gram-negative forms; and that he reached the conclusion—from serological studies—that the Gram-negative forms arose from the parent strains by loss of the ectoplasm.

REVERSAL OF REACTION OF THE COCCI

If aqueous suspensions of certain species of cocci be exposed for ten hours or more to 52°C. their Gram reaction is completely

reversed. (See figs. 1 and 3.) This change cannot be produced in all species of cocci, at least with equal ease. Of the six species studied, two, both staphylococci, were completely reversed in eighteen hours and a great proportion of the individuals, in suspensions of these organisms, had become Gram-negative much sooner than this. The staining reactions of *Rhodococcus roseus* and of *M. freudenreichii*, on the other hand, were little, if at all,

TABLE 1

	PER CENT POSITIVE			
	4 hours	5 hours	24 hours	48 hours
Staphylococcus aureus (Torrey, Richards):				
At room temperature (control).................	100	100	100	85
At 52°..................................... ..	5	0 5	9	0
Staphylococcus aureus (Torrey, No. 77):				
At room temperature (control)....	100	100	100	100
At 52°..	60	50	1	0
Staphylococcus albus (A. T. C. No. 251):				
At room temperature (control)......	100	100	100	100
At 52°.........	100	100	95	60
Staphylococcus aureus (Torrey No. 42):				
At room temperature (control).................	90	95	95	95
At 52°.....	98	98	95	95
M. freudenreichii (A. T. C. No. 407):				
At room temperature (control).............	100	100	100	99
At 52°....................................	100	100	100	100
Rhodococcus roseus (A. T. C. No. 144):				
At room temperature (control).................	100	100	100	100
At 52°......... ....:...	100	100	100	100

affected by the high temperature. The following species were studied:

    1. *Staphylococcus aureus* No. 42 ⎫
    2. *Staphylococcus aureus* No. 77 ⎬isolated by Torrey and all typical.
    3. *Staphylococcus aureus* Richards ⎭
    4. *Staphylococcus albus* A. T. C. No. 251
    5. *M. freudenreichii* A. T. C. No. 407
    6. *Rhodococcus roseus* A. T. C. No. 144

Table 1 gives the record of a typical experiment of this sort (indicated percentages refer to proportion of Gram-positive individuals in a smear).

DIMINUTION IN SIZE AND LOSS OF WEIGHT

The reversal of Gram reaction by exposure to 52° was accompanied by diminution in size (see figs. 1 and 3) and by a loss in weight. The change in size could be readily recognized in the smears; but it could not be accurately measured with the filar micrometer (as had been done in the case of *B. anthracis*) on account of the small size of the Gram-negative forms. The amount of diminution in size could, however, be accurately determined in another way. A measured amount of control bacterial suspension, and a similar amount of the same suspension in which reversal of Gram reaction had been produced, were centrifugated in two Hopkins' tubes. It was found that the

TABLE 2

	GRAM REACTION (PER CENT POSITIVE)	AMOUNT OF BACTERIA THROWN DOWN BY CENTRIFUGATION
		cc.
Specimen from ice-box..	100	0 065
Specimen boiled and kept at room temperature. .	100	0.06
Specimen kept at 52°	99	0 008

amount of bacteria thrown down in the Gram-negative tube was 87 per cent less by volume than in the control tube. The protocol of a typical experiment of this sort follows:

A suspension of *Staphylococcus aureus* (Richards), which was 100 per cent Gram-positive, was divided into three parts. One was placed in the refrigerator, a second was placed in the incubator at 52°, and a third was boiled and left at room temperature. By adopting this technic, change in bacterial volume from growth could be excluded. At the end of nineteen hours, Gram stains (Burke method) were made from each specimen, and 5 cc. of each was centrifugated for thirty minutes at high speed. The results are shown in table 2.

This change in volume was accompanied by a loss in weight. This was proven to be the case by centrifugating in previously weighed tubes, equal amounts of control (Gram-positive) and

reversed (Gram-negative) suspensions, pipetting off the supernatants, desiccating and re-weighing. Experiments of this kind showed that the reversed bacteria had lost in weight an amount which averaged about 50 per cent. A protocol of a typical experiment follows:

One hundred cubic centimeters of suspension of *Staphylococcus aureus* (Richards) was prepared. This was 100 per cent Gram-positive. It was divided into two portions of 50 cc. each. One of these was placed in the ice-box, the other in an incubator at 52°C. After an interval of twenty-four hours, the weighing experiment was done. Bacterial counts at this time showed the incubator specimen to contain 10,800,000 organisms per cubic centimeter, the ice-box specimen (control) 11,200,000 per cubic centimeter. Both bacterial growth and bacterial destruction

TABLE 3

	GRAM (PER CENT POSITIVE)	WEIGHT DESICCATED BACTERIA	LOSS WEIGHT BACTERIA	VOLUME CENTRIF-UGATE	WEIGHT DESICCATED SUPERNA-TANT	GAIN IN WEIGHT OF SUPERNA-TANT
		gram	*per cent*	*cc.*	*gram*	*per cent*
Ice-box ...	100	0 0112		0 07	0 00243	
Incubator specimen.	0	0 0056	50	0 05	0 01120	78

could, therefore, be eliminated as causes of the results obtained. Gram stains made at this time showed the ice-box specimen (control) to be still 100 per cent positive, the incubator specimen to be 0 per cent positive. Fifteen cubic centimeters of each of these suspensions (measured with Bureau of Standard pipette) were placed in previously weighed graduated centrifuge tubes and centrifugated at high speed for thirty minutes. The volume of bacteria thrown down was read off against the graduations in the tubes. Ten cubic centimeters of the supernatants were then pipetted off into previously weighed weighing bottles. Both the supernatant fluids and the centrifugated bacteria were then desiccated to dryness, and the tubes and bottles again weighed. The results are shown in table 3.

The results obtained from experiments like that just described were checked up in another way. Equal amounts of control and

reversed suspensions were passed through previously unused Berkefeld filters, which had been cleansed with aqua regia and weighed. After filtration of the bacterial suspensions, the filters were dessicated and again weighed. A loss of about 50 percent was found to have occurred in the Gram-negative forms; and a gain in weight was found to have occurred in the filtrate. Tests of the filtrates showed an increase in the amount of ninhydrin positive bodies over that obtained from the control suspensions.

<center>CONSTANCY OF THE REACTION</center>

The change of Gram reaction by exposure at 52°C. does not occur in all strains of cocci. For example, the Gram reaction of *M. freudenreichii* is entirely unaffected. On the other hand, the *Staphylococcus aureus* with which most of the experiments here reported were done—a virulent and typical organism isolated from a human being—behaved with great constancy in this respect after certain of the puzzling factors of inconstancy met with in the early experiments had been determined and taken into account. These factors were, strength of bacterial suspension, amount of suspension, and possibly the shape of the vessel in which the suspension was placed. A large series of experiments showed that constant results could be obtained, with this and a number of other strains, if (*a*) not too strong a bacterial suspension were used, and (*b*) not too large an amount of bacterial suspension. Thus when 0.05 cc. of suspension A (suspension A contained about 325,000,000 organisms per millimeter) of *Staphylococcus aureus* was placed in a pipette and exposed over night in an incubator at 52°C., absolutely constant results were obtained. Complete or nearly complete reversal of Gram reaction, with marked diminution in size, always occurred. On the other hand, if 50 cc. of suspension B (suspension B contained 20,000,000,000 mm. organisms) were placed in a 100 cc. Florence flask and similarly exposed to 52°C., reversal of Gram reaction and loss in size and weight frequently failed to occur.

It is hard to escape the conclusion from the experiments just described, that when Gram reversal is brought about by exposure of cocci to 52°, substance actually leaves the organisms whose

Gram reaction has been reversed, and that this substance passes into the fluid in which the bacteria are suspended.

EFFECT OF pH OF THE ENVIRONMENT

The well-known fact, emphasized in recent years by the Stearns (1925) and by Burke (1921) that Gram reaction is influenced by hydrogen-ion concentration, made it imperative to determine whether reversal of Gram reaction by heat could be affected by changing the pH of the environment. It was found, as a matter of fact, that the addition to the suspension of Na_2CO_3, or the corresponding Clark and Lubs buffer entirely prevented reversal of Gram reaction when the organisms were exposed to 52°. (See fig. 4.) The protocol of a typical experiment follows:

TABLE 4

	PER CENT POSITIVE	
	20 hours	120 hours
Suspension at pH 7.2:		
At room temperature..............................	100	70
At 52°.................................	0.5	0
Suspension containing 1 cc. 1 per cent Na_2CO_3 at 52° .	100	100
Suspension containing corresponding buffer at 52°.....	100	100

One-half cubic centimeter of suspension of *Staphylococcus aureus* (Richards, 100 per cent positive) was placed in each of four tubes. To two of these tubes 1 cc. of water (pH 7.2) was added, to a third 1 cc. of 1 per cent Na_2CO_3, and to a fourth 1 cc. of the corresponding buffer. (The buffer used contained 50 cc. M/5 boric acid, M/5 KCl. The whole amount diluted to 200 cc.) Gram stains were made at the end of twenty hours, and one hundred twenty hours. The results are given in table 4.

GENERAL RESULTS

The results of these experiments as to the effect of moderate heat on the Gram reaction of some strains of cocci thus parallel in every respect the results already reported as to the effect of tri-phenyl methane dyes on the Gram structure of *B. anthracis*.

Both appear to possess a Gram-positive cortex and a Gram-negative medulla. The experiments appear to offer strong evidence that the Gram structure of the two organisms is similar. The nature of the cortex in the two types must, however, differ, and causes which produce a reversal of the positivity of the cortex of one type may be without effect on the cortex of the other.

REVERSAL OF GRAM REACTION NOT A DEATH PHENOMENON

Since exposure of cocci to 52° for several hours results in their death, it might be thought that reversal of Gram reaction was simply a death phenomenon. This point was referred to in the paper on the reversal of *B. anthracis* and conclusive evidence was advanced to prove that the phenomenon could not be so simply interpreted. Nor can reversal of Gram reaction in the cocci be thus explained. If staphylococci be killed by boiling, their Gram reaction is not reversed. Nor is it reversed by exposure to acriviolet, which kills them. Reversal does not occur if alkali be added to the suspension which is to be exposed to 52°although under these conditions the organisms die. It is probably true that when dissolution of the cocci occurs, reversal of the Gram reaction also takes place (involution forms) but this is less striking a phenomenon in the case of the cocci than in the case of *B. anthracis*. Control suspensions of the latter organism after simply standing in the laboratory a few hours often contain many individual organisms whose Gram reaction has been reversed. Control suspensions of cocci, however, may stand in the laboratory for days without showing any alteration in their Gram behavior, although, of course, involution forms will finally appear and the Gram reaction be upset. That the ease with which Gram reversal occurs is a variable factor among Gram-positive organisms, that it undoubtedly takes place in some species in the normal course of growth and dissolution, and that the Gram positivity of different members of the Gram-positive group must, therefore, vary in stability, are points which have been referred to in another paper (Churchman, 1927b). The evidence of the experiments here reported, warrants the conclusion that the Gram reaction of staphylococci is more stable than

that of *B. anthracis* although based on a similar mechanism which is structural in character.[2]

MODIFICATION OF THE GRAM STAIN

In the studies on *B. anthracis* it was shown that the cortical and medullary structure of these organisms could be well revealed by modifications of the Burke stain, either in the direction of diminished exposure to methyl violet and iodide or prolonged exposure to decolorizer. Similar results may be obtained with the cocci, although the picture in this case is less striking since the organisms are so much smaller and their structure more difficult to distinguish. Certain of the cocci of which *M. freuden-reichii* may be cited as a type, appear to have so stable a Gram mechanism that it is not easily upset; the Gram reaction of this organism is not reversed by either of the methods which have produced reversal with *B. anthracis* and with certain strains of staphylococci. One cannot say postively, therefore, that these species of cocci possess the same Gram mechanisms. It seems likely that they do and that if a procedure could be found which would destroy the cortical layer, their medulla would be found also to be Gram-negative. (See figs 5 and 6.) If fixed smears of *B. anthracis* (see fig. 18) or staphylococci be exposed over night to the action of $CHCl_3$ and then stained by Burke's method, one will often (though not always) find that their Gram reaction has been reversed; and in such specimens beautiful partially positive forms of cocci are sometimes found. (See fig. 5.)

PARTIALLY GRAM-POSITIVE BACTERIA IN NATURE

During the course of these studies, a thermophilic organism grew up in a flask of agar which had been sterilized (after air contamination) only in the Arnold. This organism was a spore-bearer which grew only at 50°; and smears of it showed all variations of Gram structure, from entirely positive forms, through

[2] It should perhaps be stated, since certain claims have been made by Neide (1904) as to a staining method which he advanced, that *B. anthracis* which has been exposed to acriviolet and stained by Neide's method, has the same appearance as when stained by Burke's method. (See fig. 17.)

forms spotted with Gram-positive material on the surface to entirely Gram-negative forms. (See fig. 19.) Apparently this was an instance in nature of ·an organism which, under the conditions of culture, presented all the varieties of Gram structure which we have been able to produce artificially by exposure of *B. anthracis* to aniline dyes. It is interesting to note in this connection that, so long ago as 1875, Ferdinand Cohn (1875) in describing *Crenothrix polyspora* represents (see his fig. 21) chain-like micro-organisms surrounded by a "gallerartigen aufgequollenen Scheide." The picture is very similar to that seen in partially decolorized *B. anthracis*. Cohn's specimens were studied unstained.

OBSERVATIONS OF GUTSTEIN

In spite of a superficial resemblance between the observations of Gutstein and those reported here, and in the previous paper on *B. anthracis*, it should hardly be necessary to call attention to the fundamental difference between the picture obtained by reversal of Gram reaction and consequent denudation of medulla, and that described by Gutstein in his elaborate series of papers (1924, 1925, a, b, c, 1926). This observer, by the use of a special technic, claims in his first publication to be able to demonstrate in Gram-positive organisms, a purplish periphery and pink center. This observation he offers as evidence of a Gram-positive and Gram-negative portion for which—although the terms had previously been employed by Zettnow (1899, 1918) in a different sense—he uses the terms "ectoplasm" and "endoplasm." It is not made very clear by Gutstein (although an explanation is attempted) just how, if the intact ectoplasm completely surrounds the endoplasm, the latter gets to be stained, or if stained, how it is visible through the former.

That the evidence I have advanced for the existence of an outer Gram-positive cortex and an inner Gram-positive medulla is quite different in character from that advanced by Gutstein for an ectoplasm and an endoplasm ought to be sufficiently clear. Gutstein's evidence was tinctorial in character. Whatever interpretation be put upon his findings, it is certain that a picture

identical with that obtained by Gutstein's staining method can be obtained without the use of any pink dye at all. If staphylococci be lightly stained with methyl-violet alone; the excess of dye taken off with alcohol applied for a couple of seconds, and the smear then examined under bright illumination, the organisms appear to have a pink center and a purple periphery exactly as described by Gutstein. (Fig. 2.) The same kind of picture can be obtained by examining anthrax bacilli in a hanging drop to which a small amount of methyl-violet has been added. The axes of the organisms appear pink—their peripheries purple. This can hardly be interpreted as a tinctorial demonstration of ectoplasm and endoplasm; it is due entirely to the effect of bright light on the purple color.

In his second article Gutstein presented evidence which was more suggestive. Here he claimed to have shown, in studies on yeast cells, that so long as the cells remained Gram-positive an ectoplasm could be observed when stained by his method, whereas when the Gram reaction was reversed, no ectoplasm could be demonstrated. I have repeated these experiments with *B. anthracis*, both partly and completely reversed by acriviolet, and have been entirely unable to confirm the results of Gutstein. Schoonmaker (1924), on the basis of frozen sections of bacteria, denied the correctness of Gutstein's conclusions. Neither of these observers provided, as we have done, the evidence from volumetric, metric and weighing experiments which appears to offer the first conclusive proof of the differential structure which we have described.

Gram himself had noted that both *B. tuberculosis* and *B. anthracis* showed slender granular forms; and as long ago as 1881, Koch referred to thicker and thinner granular forms of *B. anthracis*. He did not state how the sections in which these two forms were observed had been stained, and his photographs (table VII, no. 39) were made with only 700 magnification so that the finer structures here described could hardly appear in them. Unna (1888) makes the following commentary on Koch's observation: "It is possible today to demonstrate this slender form of *B. anthracis* not only occasionally but with great regularity. This is

done by a modification of the Gram method made with that end in view; and we know definitely that the slender forms of Koch arise from the more common stout forms by a simple prolongation of the process of decolorization." In spite of the observation of Koch and the interpretation of Unna, no measurements or weighing experiments appear to have been made; and the fact that the subject was not followed up is evidence that its significance was not appreciated. That no observations considered as sufficiently definite proof of the existence of a Gram-positive and Gram-negative medulla have been previously advanced, would appear to be established by the absence of any reference to such structures in such encyclopedic works as Kolle and Wassermann (1913) and in Kolle and Hetsch (1919).

DISSOCIATION

Considering the interest aroused at the present time by the phenomenon of dissociation, it would be strange if the co-existence of Gram-negative and Gram-positive forms of *B. anthracis* in cultures had not been regarded as instances of this process. Haag (1927a and b) has offered such an interpretation of his findings. It is an easy but extremely hazardous way out of such dilemmas. For every instance of true dissociation, there are a thousand instances which are really to be explained in a simpler and more orthodox way. Since I have been able to produce Gram-negative forms of many organisms at will, and to demonstrate their structural relation to Gram-positive forms, there is no reason to appeal to dissociation to explain them.

The "gonidial forms" which Haag pictures may well be only Gram-positive material not yet detached from the surface; material of this sort which resembles the gonidia of Haag, has been pictured in my first article (1927a). That true gonidial forms do, however, occur is proven by the smear represented in figure 16. These are very different from the gonidia of Haag. They have, as can be seen, the complete structure of the bacteria (Gram-positive cortex and Gram-negative medulla) and are clearly definite buds.

It is interesting that Haag (illustration 42) shows a typical

partial decolorization in *Crenothrix polyspora*. He interprets
these structures as gonidia.

Neither of the methods used to reverse the Gram reaction of *B.
anthracis* and straphylococci produces constant results with the
yeasts. Occasionally, specimens of these organisms which are
exposed to acriviolet show a fair percentage of Gram-negative
forms (see fig. 9); but frequently when the experiment is repeated
even under what appear to be identical conditions, no effect
whatever is noted. (figs. 7 and 8.) In spite of this inconstancy,
there is a good deal of evidence to indicate that yeast cells do
possess a Gram-positive cortex and a Gram-negative medulla.
The granular appearance of the decolorizing yeast cell was first
noted by Moeller (1892) and the observation of Henrici (1914)
as to the effects produced in yeast cells by modifications of the
Gram method is well known. Henrici shows, in his black and
white illustrations, a number of acorn and girdle forms; but no
mention is made in the text of difference in size between Gram-
positive and Gram-negative forms. I am inclined to think,
although the evidence cannot yet be regarded as entirely conclu-
sive, that this spottiness is a surface phenomenon. By exposing
smears of yeast cells to stain and mordant for only a few seconds
and then decolorizing for a long period of time, or by staining and
mordanting for the usual period but prolonging decolorization,
many forms are obtained in which the Gram-negative center of the
organism appears to be enveloped by a Gram-positive outer
layer. Particularly suggestive are the numerous acorn forms,
ring forms, seal-ring forms. (figs. 10 and 11.) The most striking
picture of all is seen in the mycelial forms. Fixed smears of
yeast which have been exposed to $CHCl_3$ over night and then
stained in the usual fashion, sometimes (but not always) contain
reversed forms. (See fig. 13.)

Although the picture of partial decolorization of yeasts obtained
by modification of the Gram stain in the direction of prolonged
decolorization strongly suggests that the result is due to a solution
of outer coat, there is one difficulty about such an explanation.

This difficulty was referred to in discussing the changes produced in *B. anthracis* by modifications of the Gram stain. If the partial decolorizations are really due to an actual solution of an outer coat, one would expect to get the same result by dissolving the outer coat first with acetone ether and then carrying out the Gram stain. Under such conditions one by no means constantly gets the partial decolorization—a fact for which several explanations suggest themselves but which may really indicate that the partial decolorizations obtained by prolonged exposure of the stained organisms to acetone ether are not due to solution of cortex at all. Unfortunately, measurements with a filar micrometer—which are so helpful in enabling us to interpret the results of *B. anthracis* and which show definitely that the Gram-negative forms of this organism are actually smaller than the Gram-positive forms, are in the case of yeasts of no great help. Yeasts in any given culture differ so greatly in size that absolutely trustworthy measurements are difficult to obtain. We have, however, made a number of such measurements of the yeasts, counting a large number of cells, and have found that the average diameter of the Gram-negative forms was 16.8 per cent less than that of Gram-positive forms.

The observations of R. and W. Albert (1901) in this connection are of great interest. These authors worked with yeast cells killed by a mixture of alcohol and ether. Such dead cells were strongly Gram-positive and still retained their ability to ferment glucose. By taking samples of these dead yeast cells from the fermenting sugar solution from time to time and staining them by Gram's method, the Alberts were able to demonstrate that the cells gradually lost their power to retain the dye so that at the end of fermentation all the cells had become completely Gram-negative. Smears in the intermediate stages, however, showed a series of phenomena quite similar to those described by Henrici; that is, the cells took on a granular appearance and then became gradually fewer and fewer in number. A similar experiment in which the sugar solution was replaced by water showed that the phenomenon had no relation to fermentation, as the same changes occurred under these conditions also. The authors

were able to demonstrate further that these changes in the staining reaction of the cells went hand in hand with a process of autolysis; that as the Gram staining material became gradually lessened, the amount of coagulable albumen and of precipitate with Millon's re-agent in the supernatant fluid became greater and greater.

The authors' colored plates show stippled forms and completely negative forms like those I have produced, but no ring forms or acorns. Nothing is said about relative size.

Trommsdorff (1902) repeated these experiments and showed that the Albert phenomenon bore no relation to the content of glycogen or zymase in the cells. He further demonstrated that the loss of the Gram staining property was not due to a simple solution of some of the cell substance in water as the phenomenon failed to appear if the suspension were placed in the ice-box. Finally, he showed that if the specimen were cooked for a short time, the intracellular proteolytic enzymes being destroyed, no decolorization took place.

Crushing experiments with this organism, which lends itself readily to this type of investigation, also give suggestive results. The original observations of Benians (1912–13; 1919–20) in this field are well known. I have modified his technic so that excellent specimens of crushed yeast cells may be obtained as follows: Heavy smears of aqueous suspensions of yeast cells are made in the centers of two carefully cleansed cover slips ($\frac{3}{4}$ inch by 2 inches). These are allowed to dry, almost, but not quite, completely. The two cover slips are then placed face to face and put between two safety razor blades. These are placed between the jaws of a steel vise. The vise is clamped as tightly as possible. Even if the body weight is swung on the lever, the cover slips are not cracked. When the specimen is removed from the vise, the two cover slips will be found firmly glued together by the smears. They can be easily separated by immersing one edge in distilled water, a small amount of which will work its way between the cover clips and force them apart.

Specimens when crushed in this way will show when stained by Burke, a large number of entirely Gram-negative forms. (Fig.

12.) If the specimen be stained with methyl violet and mordanted with iodide *before crushing* and then—after crushing and fixation in the flame—be decolorized and counter-stained, large numbers of entirely Gram-negative cells will be seen; and scattered throughout the specimen will be observed Gram-positive granular material unconnected with the cells. (Figs. 14 and 15.) This is exactly the picture which one would expect if yeast cells really possess a Gram-positive cortex and a Gram-negative medulla. But it is a rather surprising fact that, when suspensions of yeast are crushed and then stained, the cells either remain entirely Gram-positive or else become entirely Gram-negative; in this type of experiment nothing can be seen of the hypothecated Gram-positive cortex which the crushing is supposed to have destroyed. This fact is perhaps not difficult to explain since the outer layer of these organisms may well be extremely tenuous and so vigorous a treatment as crushing may actually dissipate it. A dissipation of this kind may, for example, easily be observed if paramecia be suspended in a weak solution of gentian violet. The stain may be observed to percolate through the outer membrane of the organisms without much effect on it, when suddenly (probably on account of changes in surface tension) the organism appears to explode, and it may afterwards be difficult to find any trace of it.

It might seem that yeast cells would lend themselves readily to experimental study by sectioning and that their Gram structure could be readily examined in this way. I have elsewhere reported some of the difficulties which make conclusions from experiments of this kind unreliable (1928). Although we have made a great number of attempts by various technics to obtain satisfactory cross-sections of yeast cells, and some suggestive pictures have been obtained, it has been impossible to secure results in this way which give positive proof of the Gram structure of yeast cells, suggested by the other experiments.

SUMMARY

The observations with *B. anthracis* previously reported, together with those here described, justify the assumption that the

Gram reaction rests on an anatomical basis; that the essential part of all bacteria is Gram-negative; and that Gram positivity is a function of a superficial layer which the Gram-negative bacteria do not possess. This hypothesis would appear to harmonize with practically all the known facts, although some of the results obtained in the studies of yeast are perhaps not entirely consistent with it. As a working hypothesis, it would appear not unlikely that all bacteria may be placed in a Gram scale, at one end of which stand the constantly Gram-negative organisms (like *B. prodigiosus*) and at the other end the constantly Gram-positive organisms like *M. freudenreichii*. The Gram-positivity of the latter group can only be upset, if at all, with a good deal of difficulty; they remain positive in spite of treatment which reverses the positivity of other positives. At a certain place in the scale, the Gram-negative reaction disappears; beyond this point all organisms are, when examined in young cultures and by the standard method, Gram-positive. But the Gram-positivity of many of these species may be reversed; in some of them reversal is easily brought about, in others with difficulty. Some of the processes which reverse the Gram reaction of these Gram-positives, lead also to a structural change demonstrable by staining and associated with loss of size and weight. It seems altogether likely that it is this structural change which is the basis of the reversal of Gram reaction. Certain organisms (like *B. welchii* and the thermophile referred to in this paper) contain, in any given culture, some individuals which are Gram-positive, some which are Gram-negative, and some which show a Gram-negative medulla with an incomplete Gram-positive cortex. This appears to mean that some individuals of these strains develop a complete cortex, (Gram-positive forms) others fail entirely to develop a cortex (Gram-negative forms) while still others develop an incomplete rudimentary cortex (Gram variable forms). A similarly variable picture in an otherwise constantly or nearly constantly Gram-positive organism (like *B. anthracis* from young cultures) may be reproduced by exposing these organisms to acriviolet; it may be reproduced in many strains of staphylococci by exposing them to 52°. In either case, what

appears to be preponderating evidence indicates that the change in Gram reaction is due to a partial or complete destruction of the cortex. The absolutely convincing evidence for the correctness or incorrectness of this view which satisfactory cross-sections of bacteria would present, cannot now be offered since no successful technic has been developed for sectioning bacteria.

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PLATE 1

FIG. 1. Gram stain (Burke method) *Staphylococcccs aureus* (Torrey, No. 77).

FIG. 2. *Staphylococcus aureus* stained with methyl violet, lightly decolorized with alcohol, examined under high power and bright illumination. Note that, although no pink stain has been used, the organisms have pink centers exactly as described by Gutstein.

FIG. 3. *Staphylococcus aureus* after exposure for ten hours to 52°C. almost all the individuals have been reversed to Gram-negatives. Note that the Gram-negative forms are much smaller than the few remaining Gram-positives.

FIG. 4. *Staphylococcus aureus* after exposure for ten hours to 52°C. suspended in Na_2CO_3 solution. Gram reaction has not been reversed.

FIG. 5. Partial reversal in *Staphylococcus aureus* which has been exposed for twenty-four hours to $CHCl_3$.

FIG. 6. *Staphylococcus aureus* completely decolorized by prolongation of decolorization in the Burke stain.

FIG. 7. Mixture of yeasts and *B. anthracis* which has been exposed to acriviolet. *B. anthracis* has been partially reversed, yeast unaffected.

FIG. 8. Later stage in experiment represented in 7. *B. anthracis* entirely reversed; yeast unaffected.

FIG. 9. Reversal of Gram reaction of yeast produced by exposure to acriviolet.

FIG. 10. Partial decolorization of yeast produced by modification of Burke stain (diminution of staining and prolongation of decolorization).

FIG. 11. Partial decolorization of yeast produced by prolonged exposure to acetone-ether after usual exposure to stain and mordant.

FIG. 12. Crushed yeast cells, stained by Burke's method, after crushing.

FIG. 13. Reversal in yeast which has been exposed for twenty-four hours to $CHCl_3$.

FIG. 14. Yeasts stained and then crushed; no decolorizer or counter stain.

FIG. 15. Yeasts stained and then crushed; decolorization and counter-staining.

FIG. 16. Partially reversed *B. anthracis* showing "gonidial" structures.

FIG. 17. Neide stain of partially reversed *B. anthracis*.

FIG. 18. Reversal of *B. anthracis* which has been exposed to $CHCl_3$.

FIG. 19. Smear for young culture of a thermophile, showing all varieties of Gram reaction.

PLATE 1

(John W. Churchman: Gram structure of cocci.)

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